

NEUROPEPTIDES IN SMOOTH MUSCLE:
LOCALIZATION AND PARTICIPATION
IN PHYSIOLOGICAL RESPONSES

by

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in physiological responses

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This dissertation is based on the following papers which in the text will be referred to by their Roman numerals.

- I. Leander S., Håkanson R. and Sundler F.: Nerves containing substance P, vasoactive intestinal polypeptide, enkephalin or somatostatin in the guinea-pig taenia coli. Distribution, ultrastructure and possible functions. *Cell Tissue Res.* 1981; 215:21-39
- II. Leander S., Ekman R., Uddman R., Sundler F. and Håkanson R.: Neuronal cholecystikinin, gastrin-releasing peptide, neurotensin and β -endorphin in guinea-pig intestine. Distribution and possible motor functions. Submitted to *Neuroscience*.
- III. Leander S., Brodin E., Håkanson R., Sundler F. and Uddman R.: Neuronal substance P in the esophagus. Distribution and effects on motor activity. *Acta Physiol. Scand.* 1982; 115:427-437.
- IV. Tornqvist K., Mandahl A., Leander S., Lorén I., Håkanson R. and Sundler F.: Substance P-immunoreactive nerve fibers in the anterior segment of the rabbit eye. *Cell Tissue Res.* 1982; 222:467-477.
- V. Bynke G., Håkanson R., Hörig J. and Leander S.: Bradykinin contracts the pupillary sphincter and induces ocular inflammation through release of neuronal substance P. Submitted to *Br. J. Pharm.*
- VI. Leander S., Håkanson R., Rosell S., Folkers K., Sundler F. and Tornqvist K.: A specific substance P antagonist blocks smooth muscle contractions induced by non-cholinergic, non-adrenergic nerve stimulation. *Nature* 1981; 294:467-469
- VII. Håkanson R., Hörig J. and Leander S.: The mechanism of action of a substance P antagonist ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP. *Br. J. Pharm.* 1982; In press.
- VIII. Håkanson R., Leander S., Andersson R.G.G. and Hörig J.: Substance P antagonists release histamine from peritoneal mast cells. Submitted to *Acta Physiol. Scand.*



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Contents

	<u>Page</u>
INTRODUCTION	9
Aim of the present study	12
METHODS	13
Immunocytochemistry	13
Radioimmunochemical analyses	16
Electron microscopy	16
Studies on the motor activity of smooth muscle	16
Procedure for studying inflammatory responses in the rabbit eye	18
Procedure for studying histamine release from isolated mast cells	19
RESULTS	20
Studies on the occurrence and distribution of peptide-containing nerve fibers	20
Neuropeptides in the smooth muscle of the guinea-pig intestine	20
Neuronal SP in guinea-pig, cat and pig esophagus	22
Neuronal SP in the rabbit eye	23
Pharmacological evidence for non-cholinergic, non-adrenergic nerves in smooth muscle	23
Substance P	25
Vasoactive intestinal polypeptide	25
Leu- and met-enkephalins	25
Somatostatin	25
Cholecystokinin-octapeptide	25
Gastrin releasing peptide and bombesin	26
Neurotensin	26
β -Endorphin	26
Studies on the possible transmitter role of SP	26
Effects of (D-Pro ² , D-Trp ^{7,9})-SP in different smooth muscle systems	26
Analysis of the smooth muscle contraction produced by (D-Pro ² , D-Trp ^{7,9})-SP	29
Role of SP in ocular inflammation	30

DISCUSSION	31
Topography of peptide-containing nerve fibers	31
Origin of peptide-containing nerves in the gut	33
Projections of peptide-containing neurones in the gut	33
Ultrastructural localizations of neuropeptides in the gut	34
Are the neuropeptides transmitters?	35
Motor effects of the neuropeptides	36
Is SP a neurotransmitter?	38
Are VIP, enkephalin, somatostatin, GRP, β -endorphin, neurotensin and CCK neurotransmitters?	40
SP and ocular inflammation	40
SUMMARY	42
ACKNOWLEDGEMENTS	43
REFERENCES	44

Introduction

Attempts to elucidate the mechanisms behind different bodily functions have traditionally relied heavily on in vitro studies of isolated tissue preparations. Smooth muscle preparations have been particularly helpful in the analysis of the function and organisation of the autonomic nervous system.

Based on studies by Langley (1898, 1905) the autonomic nervous system was divided into one sympathetic and one parasympathetic part. Subsequently noradrenaline was identified as the transmitter at sympathetic and acetylcholine at parasympathetic effector junctions. Already in 1898 Langley observed an atropine-resistant relaxation of the stomach after vagal nerve stimulation, and later it was shown that also adrenergic blocking drugs were without effect (Greeff et al. 1962), suggesting the involvement of nerves other than cholinergic and adrenergic ones. Also in other tissues, such as the guinea-pig urinary bladder, neuronal responses have been observed that could not be explained within the traditional concept of either acetylcholine - or noradrenaline-mediated effects (Langley and Andersson 1895; Henderson and Reopke, 1934, 1935; Ambache, 1955). Attention was again focused on non-cholinergic, non-adrenergic neuronal mechanisms in the early 1960's when Burnstock demonstrated relaxatory as well as contractile non-cholinergic, non-adrenergic responses in the isolated guinea pig taenia coli (Burnstock et al. 1963a, b, 1964). These responses were blocked by the neuronal blocker tetrodotoxin (TTX)* indicating a neuronal involvement. Since then interest in nerve-mediated non-cholinergic, non-adrenergic responses has grown steadily (see for example reviews by Furness and Costa, 1973; Burnstock and Szurszewski, 1979). Apart from electrophysiological and pharmacological evidence (Burnstock 1972) for non-cholinergic and non-adrenergic neuronal mechanisms a number of observations indicates the existence of nerves, morphologically distinct from adrenergic and cholinergic nerves (p-type nerves) (Baumgarten et al. 1970; Cook and Burnstock, 1976). The term p-type derives from the fact that the ultrastructure of these nerve fibers is reminiscent of the peptide-containing, neurosecretory nerve fibers of the posterior pituitary. Studies on the fine-structural details of the vesicles in p-type nerve terminals have indicated the existence of several subpopulations of such nerves (Cook and Burnstock, 1976).

* Tetrodotoxin, a neurotoxin obtained from the puffer-fish, is a potent blocker of neuronal conduction acting by inhibiting inward sodium currents (Kao, 1966).

In a series of publications Burnstock and co-workers (Burnstock 1972, 1975, 1979; Burnstock and Bell, 1974) have argued that at least a proportion of the p-type nerve fibers, notably those containing large opaque vesicles, are purinergic, i.e. they are thought to employ purine nucleotides, adenosine triphosphate (ATP) in particular, as transmitter. In the early experiments carried out to identify the transmitter in the non-cholinergic, non-adrenergic nerve fibers of the gut, ATP was thought to satisfy the criteria for a neurotransmitter (Burnstock, 1972). (1) Both ATP and the enzyme systems that synthesize ATP seem to occur in nerve cells. Tritium-labeled adenosine is taken up by specimens of the gut wall and rapidly converted to ATP (Su et al., 1971). Most of the labeled ATP is stored in nerves (see Burnstock, 1972). (2) Stimulation of enteric nerve fibers after loading with tritiated adenosine results in release of tritium (Su et al., 1971; Kuchi et al., 1973). This release is reduced by tetrodotoxin (Su et al., 1971). (3) Smooth muscle is highly sensitive to adenine nucleotides. The response to exogenous ATP resembles that to stimulation of non-cholinergic, non-adrenergic nerves (see, for instance, Nakanishi and Takeda, 1972, 1973; Davison et al., 1978). Other transmitter candidates were rejected as contenders on the grounds that they were either inactive or did not mimic the nerve-mediated responses; that specific blocking drugs for these substances did not affect the nerve-mediated response; or that their action was mediated indirectly through stimulation of nerve fibers and not by a direct effect on smooth muscle (cf. Burnstock, 1975). Although the existence of a non-cholinergic, non-adrenergic neuronal system is generally accepted, the proposal that ATP is the transmitter is not (see, for instance, Rikimaru et al., 1971; Ambache et al., 1977; Bloom and Polak, 1978; Humphrey and Fischer, 1978; Jones, 1978; Small and Weston, 1979; Håkanson et al., 1981).

Burnstock's concept has undergone little modification since 1972, when it was first introduced. In the last five or six years, many other putative neurotransmitters have appeared that are competing with ATP for a transmitter role. Peptides in particular have attracted much interest. The number of peptides found in neuronal elements is rapidly increasing. Many brain peptides have been demonstrated in the periphery, in nerves or in endocrine cells, or in both. Interestingly, many peptides known or thought to be hormones have been detected in the brain and in nerve fibers in the periphery (cf. Polak and Bloom, 1978; Emson, 1979; Hökfelt et al., 1980; Sundler et al., 1980) (Table 1). The gut is particularly rich in peptide-containing nerve fibers, most of which seem to be of intrinsic origin (Malmöfors et al. 1980, 1981). Much work has been devoted to their distribution (Schultzberg et al., 1980), polarities and projections (Furness and Costa, 1979,

Table 1

Amino-acid sequences of known peripheral neuropeptides.

Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂
Vasoactive intestinal polypeptide	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Glu-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH ₂
Gastrin Releasing Peptide	Ala-Pro-Val-Ser-Val-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH ₂
Met-Enkephalin	Tyr-Gly-Gly-Phe-Met
Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu
Somatostatin	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
β-Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Cholecystokinin-8	Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH ₂ SO ₃
Neurotensin	pGlu-Leu-Thr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu

* Gastrin releasing peptide represents the mammalian counterpart of amphibian bombesin.

1980; Costa et al., 1980b, 1981).

This presentation will deal with the following peripheral neuropeptides: substance P (SP), vasoactive intestinal polypeptide (VIP), enkephalin, somatostatin, cholecystokinin (CCK), gastrin-releasing peptide (GRP), neurotensin and β -endorphin (Table 1). Their possible roles as neurotransmitters have been analysed with respect to their localization in nerve fibers, bio-activity profiles and effects of specific peptide receptor blocking agents.

Aim of the present study

The aim of the present investigation is to define the occurrence, distribution and functional roles of eight different neuropeptides in the periphery with special reference to their involvement in the control of smooth muscle activity. The following topics were studied:

1. The occurrence and distribution of SP, VIP, enkephalin, somatostatin CCK, GRP, neurotensin and β -endorphin in the guinea-pig intestine and of SP in the cat esophagus and rabbit eye.
2. The ultrastructure of nerve fibers containing SP, VIP and enkephalin in the guinea-pig taenia coli.
3. The effects of the above mentioned neuropeptides on basal tension and electrically induced responses in the guinea-pig taenia coli and of SP on basal tension and electrically induced responses in the cat esophagus and rabbit sphincter pupillae muscle.
4. The effects of bradykinin on the rabbit eye in vivo and on the rabbit sphincter pupillae muscle in vitro.
5. The functional significance of neuronal SP in the smooth muscle of the guinea-pig taenia coli, cat esophagus, rabbit sphincter pupillae muscle and guinea-pig bladder as studied by means of a specific SP antagonist.
6. The mode of action of SP antagonists.

Methods

Immunocytochemistry (I, II, III, IV)

Antisera against SP, VIP, leu- and met-enkephalin, somatostatin, GRP, neurotensin and β -endorphin (Table 2) were used to visualize neuronal elements. The tissues examined included the digestive tract of guinea pig, the oesophagus of guinea pig, cat and pig, and the uvea of the rabbit. The tissues specimens were processed for immunocytochemistry in different ways: 1) Specimens were freeze-dried, vapour-fixed with diethylpyrocarbonate and paraffin-embedded. 2) Specimens were fixed by immersion overnight in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2. After rinsing in sucrose-enriched buffer, the specimens were frozen on dry ice and sectioned in a cryostat. 3) Specimens were collected from animals perfused via the heart with an ice-cold solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2; the specimens were immersed in the fixative for 10-12 hours and rinsed in sucrose-enriched buffer. They were then frozen and sectioned in a cryostat. 4) Whole mounts were prepared from intestinal smooth muscle or irides by stretching on microscope slides. The whole mounts were then fixed in a solution of picric acid and formaldehyde, dehydrated, cleared in xylene and hydrated. The sections and whole mount preparations were exposed to peptide antisera in the appropriate dilution at room temperature for 3 hours in a moist chamber. Following thorough rinsing of the sections the sites of the antigen-antibody reaction were visualized by the indirect immunofluorescence method of Coons et al. (1955) or by the peroxidase-antiperoxidase (PAP) method of Sternberger (1979). Controls were as follows: 1) the peptide antiserum was omitted; 2) the second antibody was omitted; 3) normal rabbit serum was applied instead of peptide antiserum; 4) each peptide antiserum was permitted to react with synthetic antigen (10-100 μ g/ml diluted antiserum) for at least 24 hours in the refrigerator before being applied to the sections. Such absorption of the antisera resulted in elimination of specific immunoreaction.

Each antiserum was tested for cross-reactivity with other neuropeptides so that its specificity could be assessed. Cross-reactivity with peptides or proteins containing amino acid sequences similar to that of the immunoreactant cannot be excluded. It would therefore be appropriate to refer to the immunoreactive material as SP-like, VIP-like etc. For brevity however, the immunoreactive nerve fibers are referred to as SP fibers, VIP fibers etc. Immunocytochemistry cannot be used to identify unequivocally the type of molecule visualized. For this purpose immunochemical analysis together with chromatographic separation is necessary.

Table 2

Details of the antisera.

Antigen	Code	Paper	Raised against	Directed against	Working dilution Immuno- PAP fluoresc. staining	Source	References
1. SP	SP-8	I, III, IV	protein-conj. bovine substance P	C-terminus	1:160 1:80	P. Emson, Cambridge University, Cambridge England	Brodin et al. 1981
	K-25	III	protein-conj. bovine substance P	C-terminus	1:40	E. Brodin Karolinska Institutet Stockholm, Sweden	Brodin et al. 1981
	JK-1	IV	protein-conj. bovine substance P	C-terminus	1:320	P. Emson, Cambridge University, Cambridge England	Brodin et al. 1981
2. VIP	5603	I	protein-conj. porcine VIP	C-terminus	1:80	J. Fahrenkrug Bispe- bjerg Hospital, Copen- hagen, Denmark	Larsson et al. 1976
3. Leu-Enk	170	I	protein-conj. leu-enkephalin	C-terminus	1:60	K-J Chang, Burroughs Wellcome Research Triangle PARK, NC USA	Alumets et al. 1978
4. Somato- statin	19578	I	protein-conj. somatostatin	C-terminus	1:80	M.P. Dubois, Institut National de Recherche Agriculture, Nouzilly, France	Dubois 1975
5. Gastrin	4562	II	protein-conj. human gastrin 2-17	C-terminus	1:640	J.F. Rehfeld Aarhus, Denmark	Lorén et al. 1979

6. GRP	6902	II	protein-conj. porcine GRP	C-terminus	1:320	1:5120	N. Yanaihara, Shizouka Med.Coll. Zhizouka, Japan	Yanaihara et al. 1981
7. Neuro- tensin	HC-8	II	protein-conj. bovine neurotensin	C-terminal hexapeptide	1:40	1:160	R.E. Carraway, Boston Mass., USA	Sundler et al. 1977a
8. β -End.	7762	II	unconj. β -endorphin	NH ₂ -terminus	1:40	1:80	own	Brammert et al. 1982
	7763	II	unconj. β -endorphin	C-terminus	1:40	1:80	own	Brammert et al. 1982

Comments

1. All SP-antisera cross react with other tachykinins such as physalaemin and eledoisin. SP-8 and JK-1 do not cross-react with bombesin or GRP. K-25 cross reacts with bombesin and GRP.
3. This antiserum cross-reacts with met-enkephalin.
5. This antiserum recognizes the COOH-terminal pentapeptide common to gastrin and CCK and does not discriminate between gastrins and CCKs at the immunocytochemical level.
6. This antiserum is directed against the COOH-terminal portion of GRP and when used for immunocytochemistry recognizes also bombesin (Yanaihara et al., 1981).
7. This antiserum recognizes the COOH-terminal hexapeptide of neurotensin in a radioimmunoassay system (Carraway and Leeman, 1976a, b).
8. 7762 is probably directed to the C-terminal part of β -endorphin. It does not cross react with β -lipotropin. 7763 is directed to the N-terminal part of β -endorphin and cross reacts with β -lipotropin.

Radioimmunochemical analyses (II)

SP, GRP, neurotensin and β -endorphin were determined by radioimmunoassay. In each case, the identity of the immunoreactive material was verified by 1) comparing the dilution curve of the extracts with that of the standard, and 2) chromatographic analysis of the immunoreactive material. In the case of SP and β -endorphin, analysis was by gel chromatography, in the case of GRP and neurotensin the analysis was by high performance liquid chromatography.

Electron Microscopy (I)

Tissue specimens were immersed in fixative consisting of 0.5% glutaraldehyde and 3.5% formaldehyde in 0.075 M phosphate buffer, pH 7.2, for 1 to 4 h. They were postfixed in 1% OsO_4 for 2 h, dehydrated in graded ethanol solutions, contrasted en bloc for 1/2 h in a mixture of 1% phosphotungstic acid and 0.5% uranyl acetate in ethanol and embedded in Epon. Ultrathin sections were exposed to the antisera against SP, VIP, enkephalin or somatostatin. The site of antigen-antibody reactions was revealed by the PAP procedure (see above). For details see Alumets et al. (1977, 1978).

Studies on the motor activity of smooth muscle (I, II, III, IV, V, VI, VII)

Investigations were performed on isolated preparations of guinea-pig taenia coli (I, II, VI, VII) (Fig. 1), cat esophageal circular smooth muscle (III), rabbit iris sphincter pupillae muscle (IV, V, VI) and guinea-pig urinary bladder (VI). The preparations were mounted vertically on perspex holders in 7-10 ml organ baths maintained at 33-37°C. One end of the preparation was attached to a rigid support and the free end to a lever connected via a spring to a Grass FT 03 force displacement transducer or to a photoelectric transducer for isotonical registration of mechanical activity. The load on the preparations was usually set at 0.2 g. Platinum ring electrodes were placed on either side of the preparations with a constant electrode distance of 5 mm. The electrodes were connected to a Grass S 4C stimulator for field stimulation with square wave pulses (10-15 V over the electrodes 0.5-1 ms duration). The preparations were stimulated with trains of pulses with a resting period between each train. The mechanical activity of the preparations was recorded continuously throughout the experiments. The bathing fluid was a modified Krebs solution, bubbled with a gas mixture of 7% CO_2 in O_2 giving a pH 7.2-7.3. The preparations were allowed to equilibrate in the bath for 60 min before experimentation started. Concentration-response curves were constructed either by adding step-wise increasing amounts of peptides to the bath or by testing only one concentration on

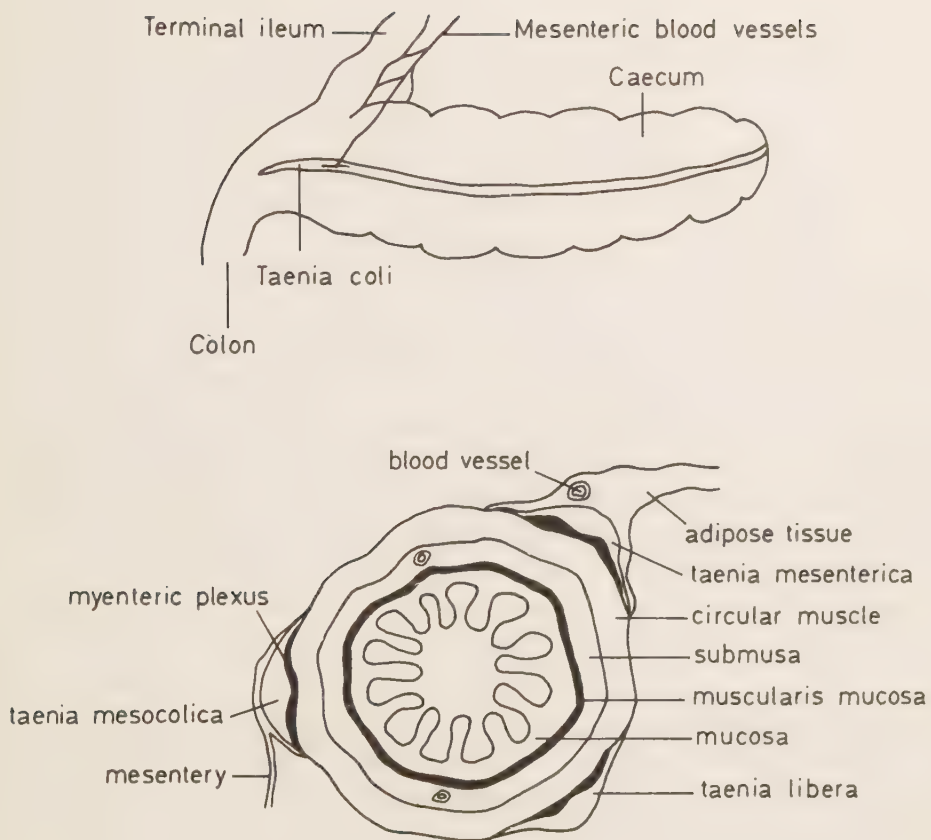


Fig. 1. Top. The guinea-pig caecum and the taenia coli situated on the caecum. Below. A transverse section of the caecum.

each preparation.

Isotonic recordings offer certain advantages over isometric recordings. Isotonic conditions can be characterized in terms of the load applied to the tissue. An isotonic system enables the muscle to alter its length freely, whereas an isometric system offers limited freedom for changes in length. However, there are certain problems associated with isotonic recordings. There is an arbitrary element involved in the choice of load for a given preparation. As the motor activity is greatly dependent upon the load this must be selected so that stable and reproducible conditions are obtained with respect to basal tension, spontaneous activity and response to stimuli. The load must ensure a clear difference between the amplitude of the spontaneous activity and the amplitude of evoked contractions. The effect of different loads on the spontaneous activity of the guinea-pig taenia coli has been studied in detail by Ishida and Urakawa (1974). They found that at a load of 0.2 g the taenia displayed a regular spontaneous activity. As the load was increased the spontaneous activity approached that observed during isometric conditions where periods of no activity are interrupted by bursts of activity. In the present study we found a load of 0.2 g to enable the taenia to respond to inhibitory as well as excitatory signals with reproducible responses. The other preparations tested responded optimally at a load of 0.2 g, or 0.5 g for cat esophagus preparations.

Procedure for studying inflammatory responses in the rabbit eye (V)

Adult pigmented rabbits were used. They were anesthetized with methohexital sodium (Brietal, Lilly, 5 mg/kg) when given injections into the eyes. No anesthesia was administered during the rest of the experiments. The blood-aqueous barrier of the eye was disrupted by intravitreal injection of SP or bradykinin. The time course of the barrier damage was followed by photoelectric measurement of the aqueous flare response (AFR) (Bengtsson 1975). This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and the protein concentration has been established (Anjou and Krakau 1961). The results are expressed in arbitrary units with reference to a standard. The AFR and the pupillary diameter were measured every 30 min. Bradykinin (350 pmole in 15 μ l), SP (3 nmole in 9 μ l) or 0.9 percent saline (9 or 15 μ l) was injected into the corpus vitreum. Atropine 1% and the SP antagonist (Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH₂) were applied topically in a volume of 60 μ l to the left eye. Atropine was administered 1 hour before injection of bradykinin. The SP antagonist was given twice, 1 hour and 10 min before bradykinin. If not otherwise stated, the right eye re-

ceived 0.9 percent saline topically before injection of bradykinin. TTX was administered by intravitreal injection (15 nmole in 10 μ l) 4 hours prior to injection of bradykinin.

Procedure for studying histamine release from isolated mast cells (VIII)

Adult male Sprague-Dawley rats were killed by decapitation under light diethyl ether anaesthesia. Peritoneal cells, consisting of 3-6% mast cells (Padaver, 1963; Kurose and Saeki, 1981). were obtained by peritoneal lavage with 10 ml buffered salt solution, containing 145 mM NaCl, 2.7 mM KCl and 10% (v/v) Sörensen phosphate buffer. After gentle abdominal massage for 3-4 min, the intra-abdominal mast cell-rich fluid was removed with a pipette. Abdominal fluid from 2 rats was pooled and centrifuged. The precipitated cells were resuspended, washed and divided in aliquots of 0.9 ml. The release of histamine from these cells upon exposure to SP or one of the two SP antagonists ($\underline{\underline{D}}$ -Pro², $\underline{\underline{D}}$ -Trp^{7,9})-SP or Arg-Gln- $\underline{\underline{D}}$ -Trp-Phe- $\underline{\underline{D}}$ -Trp-Leu-Met-NH₂ was measured by a direct fluorometric method (Håkanson and Rönnberg 1974). The amount of histamine released was expressed as per cent of the total amount of histamine present in the peritoneal cells.

Results

Studies on the occurrence and distribution of peptide-containing nerve fibers

Neuropeptides in the smooth muscle of the guinea-pig intestine (I, II). SP, VIP, enkephalin, somatostatin, CCK, GRP, neurotensin and β -endorphin could be demonstrated in nerve fibers in the myenteric plexus and in the smooth muscle. Generally, peptide-containing nerve fibers were more numerous in the ileum and colon than in the duodenum and jejunum. They were all seen to run in thin nerve bundles and as single fine-varicose terminals along the longitudinal smooth muscle bundles (Fig. 2). However, the different types of nerve fibers had somewhat different regional and topographic distribution (Fig. 3). Nerve fibers containing immunoreactive SP, VIP, enkephalin, GRP and β -endorphin were quite numerous in the smooth muscle while those containing somatostatin, CCK and neurotensin were few (Fig. 3).

Fig. 2

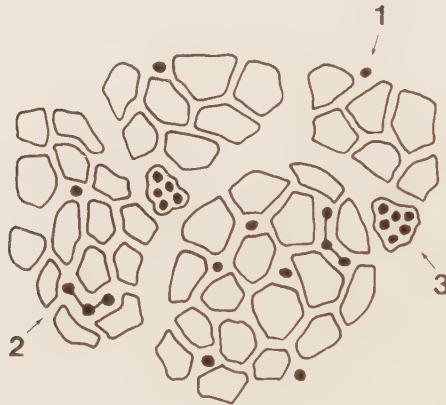


Fig. 2. Schematic representation of smooth muscle innervation. The figure shows a transverse section through muscle cells gathered in bundles. 1: Nerve ending or axon varicosity. 2. Successive axon varicosities of one axon. 3. Nerve bundle.

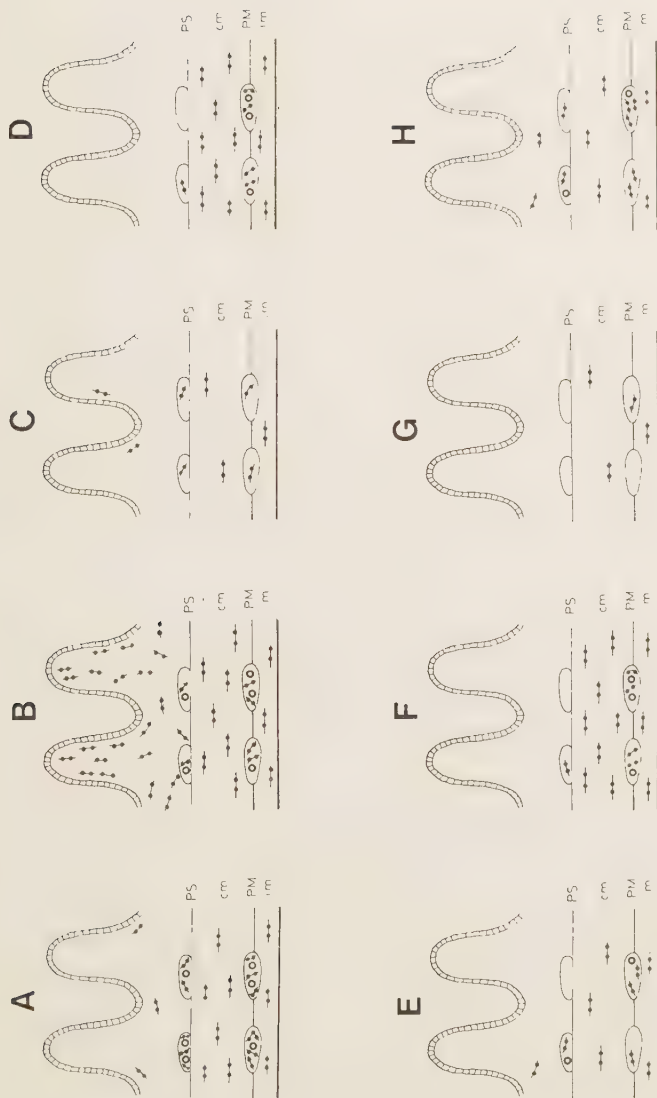


Fig. 3. Schematic outline of the topographical distribution and relative frequency of nerve fibers (—●—) and nerve cell bodies (o) displaying SP (A), VIP (B), somatostatin (C), enkephalin (D), CCK (E), β -endorphin (F), neurotensin (G) and GRP (H) immunoreactivity. Data are from guinea-pig intestine. PS = submucosal plexus, PM = myenteric plexus, Cm = circular muscle, lm = longitudinal muscle.

In addition, rather many nerve fibers within the myenteric plexus displayed SP, VIP, enkephalin, β -endorphin or GRP immunoreactivity. CCK fibers were moderate in number while somatostatin and neurotensin fibers were few. Nerve cell bodies containing immunoreactive SP were numerous while VIP- and β -endorphin-containing cell bodies were moderate in number. Only few cell bodies displaying enkephalin, CCK or GRP immunoreactivity could be detected and somatostatin or neurotensin immunoreactive cell bodies were not seen at all. Nerve fibers containing SP, VIP, enkephalin, somatostatin or GRP were seen to ramify around nerve cell bodies in the plexus. HPLC of extracts of intestinal smooth muscle revealed one major neurotensin- and one major GRP-immunoreactive compound that co-eluted with authentic neurotensin and GRP, respectively. Gel chromatography of extracts of guinea-pig ileum revealed one major β -endorphin-immunoreactive peak with the same elution position as synthetic β -endorphin.

Nerve fibres characterized ultrastructurally by the presence of large dense-cored vesicles were numerous in the myenteric plexus. Such fibres were regularly seen also in the smooth muscle. By immunostaining ultrathin sections it was possible to show that three peptides (SP, VIP or enkephalin) were stored in such vesicles. The various types of peptide-containing nerve fibres were ultrastructurally rather similar.

Neuronal SP in guinea-pig, cat and pig esophagus (III). Fine varicose SP-immunoreactive nerve fibres were observed in the esophageal smooth muscle layers of all species studied. Of the two antisera tested one cross-reacted with bombesin. Both showed the same distribution and frequency of immunoreactive nerve fibres suggesting that the immunostaining reflected the presence of SP (or a closely related peptide). Immunoreactive nerve fibres were fairly numerous in the guinea-pig and cat but few in the pig. They were more numerous in the circular than in the longitudinal smooth muscle and the muscularis mucosae. Immunoreactive fibres did not seem to be associated with the striated muscle that predominated in the proximal parts of the esophagus. Striated muscle was found in the upper 2/3 of the esophagus in the cat and in the entire esophagus, but for a narrow ring distally, in the other species. SP-immunoreactive fibres were numerous in the myenteric and submucosal plexuses, often surrounding nerve cell bodies that did not display SP immunoreactivity. SP fibres occurred around small blood vessels along the entire esophagus including those in striated muscle. Only in the cat esophagus were SP-immunoreactive nerve cell bodies detected. Such cell bodies were few and restricted to the myenteric plexus. The lower

part of the cat esophagus contained approximately 10 times more immunoreactive SP than the upper part, while the middle part contained intermediate amounts. In all regions examined, the muscle layer of the esophageal wall contained more SP than the mucosa.

Neuronal SP in the rabbit eye (IV). A moderate to low number of SP-immunoreactive nerve terminals were found in the anterior uvea. In the iris, immunoreactive fibres were seen to run immediately in front of the dilator muscle, within the sphincter muscle and close to blood vessels. The SP fibres did not seem to penetrate into the dilator muscle. In the sphincter muscle they were most numerous in the part opposing the pupillary margin. The iris stroma contained a moderate number of SP fibres forming a loose plexus. SP fibres were more numerous around the larger blood vessels than around the smaller ones. Immunoreactive fibres were rare in the trabecular tissue of the chamber angle; only occasionally were short SP fibres seen near the aqueous channels in the sclera. The ciliary body contained very few SP fibres, without obvious relation to the ciliary muscle. The anterior ciliary processes often contained varicose SP fibres, which at times could be followed over long distances. They had a subepithelial location, often interposed between blood vessels and the epithelial lining (Tervo et al., 1980, 1981). The posterior ciliary processes contained SP fibres infrequently. The cornea displayed a disturbingly high background fluorescence (autofluorescence and unspecific binding of antiserum); no SP fibres could be demonstrated in the cornea. The sclera contained extremely few fibres. All antisera gave identical results.

Pharmacological evidence for non-cholinergic, non-adrenergic nerves in smooth muscle

In all the smooth muscle preparations studied electrical field stimulation produced non-cholinergic, non-adrenergic neuronal responses (Fig. 4). They could be revealed by addition of a cholinergic blocker (atropine) and an adrenergic blocker (guanethidine). TTX-sensitive responses to electrical field stimulation under these circumstances represented non-cholinergic, non-adrenergic neuronal mechanisms (Fig. 4): in the taenia coli a relaxation followed by a contraction, in the cat esophagus a relaxation, in the guinea-pig urinary bladder a contraction and in the rabbit sphincter pupillae a contraction. If the neuropeptides were to be considered as transmitter candidates they should be able to mimic these responses.

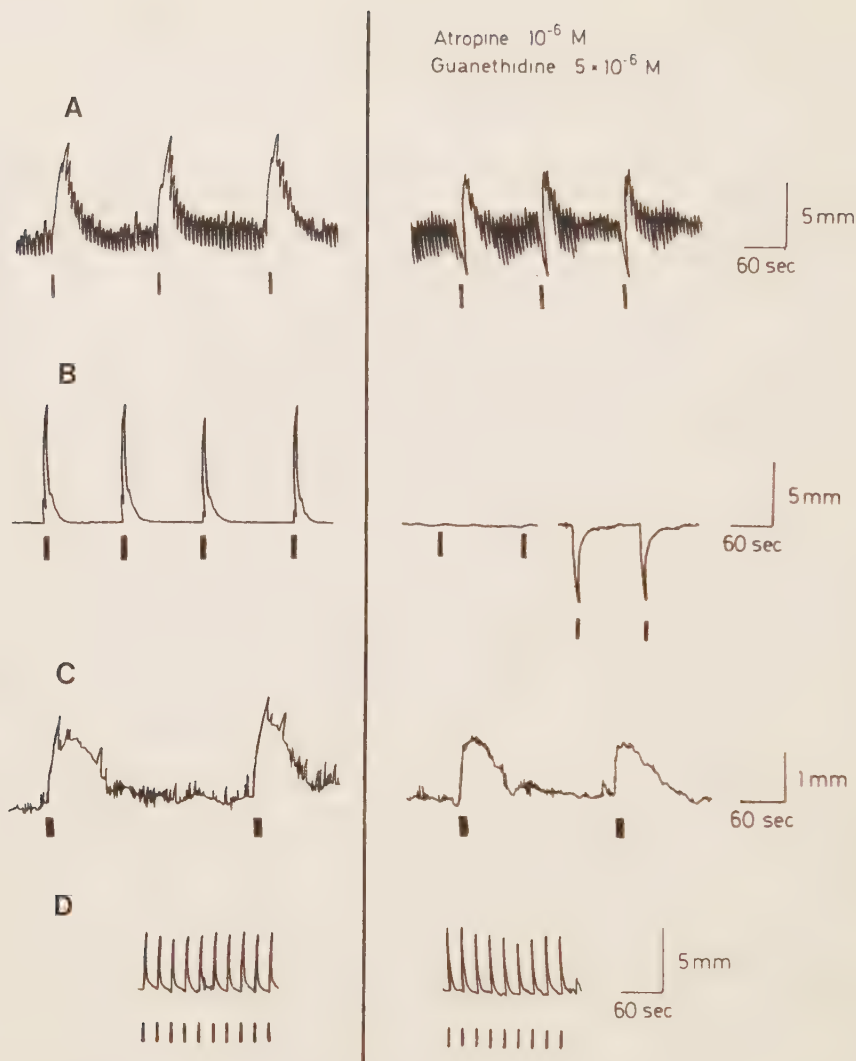


Fig. 4. Typical tracings showing the effect of electrical stimulation on control preparations (left) and in the presence of atropine and guanethidine (right). The preparations were stimulated when indicated by black rectangles. A. Guinea-pig taenia coli (3 Hz, 3 sec). B. Cat esophageal circular smooth muscle (5 Hz, 3 sec, for relaxation: 1 Hz, 3 sec). C. Rabbit sphincter pupillae muscle (30 Hz, 10 sec). D. Guinea-pig urinary bladder (1 pulse/20 sec). Note the presence of non-cholinergic, non-adrenergic contractile responses in A, C and D and relaxatory responses in A and B. All these responses could be abolished by TTX (10^{-6} M).

Substance P (I, III, IV, V, VI, VII). SP contracted the isolated guinea-pig taenia and the circular smooth from cat esophagus in a concentration dependent manner (10^{-9} - 10^{-5} M). The rabbit sphincter pupillae muscle responded to SP (at concentrations above 10^{-9} M) with a long lasting contraction. All these responses were unaffected by cholinergic and adrenergic blocking drugs or by TTX. In the SP contracted taenia the electrically induced contractions were reduced in amplitude while the relaxations seen after addition of atropine + guanethidine were enhanced in amplitude. In concentrations (10^{-8} - 10^{-7} M) that caused a slight increase in basal tension of the cat esophageal smooth muscle, SP greatly enhanced the contractile response to electrical field stimulation. Also the myogenic responses obtained upon high frequency electrical stimulation (30 Hz, 10 s, 1 ms) of TTX-treated preparations were enhanced by SP.

Vasoactive intestinal polypeptide (I). VIP relaxed the taenia in a concentration dependent manner (10^{-9} - 10^{-6} M). This response was not affected by cholinergic or adrenergic blocking drugs or by TTX. In a taenia preparation relaxed by VIP an increased contractile response to electrical stimulation could be observed.

Leu- and met-enkephalins (I, III). The enkephalins, in concentrations above 10^{-6} M, raised the resting tension of the taenia slightly. The resting tension of cat esophageal smooth muscle was not affected. Concentrations of enkephalins above 10^{-6} M slightly reduced the electrically evoked atropine sensitive contractile response in the taenia. This effect was not blocked by naloxone. Electrically evoked atropine sensitive contractions in the cat esophageal smooth muscle could be abolished by leu-enkephalin in concentrations above 10^{-7} M. This inhibitory effect of leu-enkephalin was blocked by naloxone.

Somatostatin (I). Somatostatin in concentrations up to 10^{-5} M did not appreciably affect the resting tension of the taenia. A very small reduction of the amplitude of electrically induced responses could be observed both in control and cholinergically and adrenergically blocked preparations.

Cholecystokinin-octapeptide (II). CCK-8 caused a concentration-dependent (10^{-9} - 10^{-7} M) increase of the tension of the taenia. Atropine reduced the maximum contraction by approx. 20% and TTX by 45%. CCK-8 increased the amplitude of electrically evoked responses both in the presence and absence of atropine.

Gastrin releasing peptide and bombesin (II). GRP and bombesin produced concentration-dependent (10^{-9} - 10^{-7} M) contractions in the taenia coli that could not be blocked by either atropine or TTX. These peptides also reduced electrically induced contractile responses in the taenia but only in concentrations that raised the resting tension. The amplitude of the relaxation was not affected.

Neurotensin (II). Also neurotensin produced concentration-dependent (10^{-11} - 10^{-6} M) contractions in the taenia coli that could not be blocked by atropine or TTX. Neurotensin reduced the amplitude of electrically evoked contractions but only in concentrations that raised the resting tension. The amplitude of the relaxation was not affected.

β -Endorphin (II). β -Endorphin had no effect on the resting tension of the taenia coli. However, β -endorphin greatly reduced both the cholinergic and the non-cholinergic contraction in the taenia via a naloxone sensitive mode of action. The relaxation was not affected.

Studies on the possible transmitter role of SP

Analysis of the physiological roles of the neuropeptides requires specific blocking substances. It was therefore of interest to use the recently developed SP antagonists (Folkers et al., 1981; Holmdahl et al., 1981; Rosell et al., 1981). SP and related peptides (so-called tachykinins) share amino acid sequences, that seem to be essential for their biological activity (Table 3). The design of SP antagonists was based on the substitution of the conserved amino acids in the tachykinin group of peptides. The two antagonists used are shown in Table 3.

Effects of (D-Pro², D-Trp^{7,9})-SP in different smooth muscle systems (VI).
The addition of SP and of (D-Pro², D-Trp^{7,9})-SP to quinea pig taenia coli produced a contraction which lasted 120-150 s, the tension subsequently returning to baseline. The SP analogue evoked contraction only at concentrations above 10^{-6} M. The contractile response to exogenous SP was strongly inhibited by pretreatment with (D-Pro², D-Trp^{7,9})-SP, the blockade lasting for at least 30 min without washing. Washing out the SP analogue resulted in a gradual return of the contractile response to exogenous SP and to electrical stimulation. The analogue reduced the contractile response to exogenously applied SP-related peptides (physalaemin and eledoisin) but not that to carbachol, histamine, serotonin, bradykinin, Lys⁸-vasopressin or prostaglandin F_{2 α} (Fig. 5). In the presence of (D-Pro², D-Trp^{7,9})-SP,

Table 3

Amino acid sequences of tachykinins* and of two SP antagonists. Amino acids common to all tachykinins are boxed.

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂	Substance P
Pyr-Pro-Asp-Pro-Asn-Ala-Phe-Tyr-Gly-Leu-Met-NH ₂	Uperolein
Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂	Physalaemin
Pyr-Asn-Pro-Asn-Arg-Phe-Ile-Gly-Leu-Met-NH ₂	Phyllomedusin
Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH ₂	Eledoisin
Asp-Val-Pro-Lys-Ser-Asp-Glu-Phe-Val-Gly-Leu-Met-NH ₂	Kassinin
Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH ₂	SP antagonist
Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH ₂	SP antagonist

* SP and related peptides isolated from various non-mammalian species (Erspamer and Melchiorri, 1973; Erspamer et al., 1977).

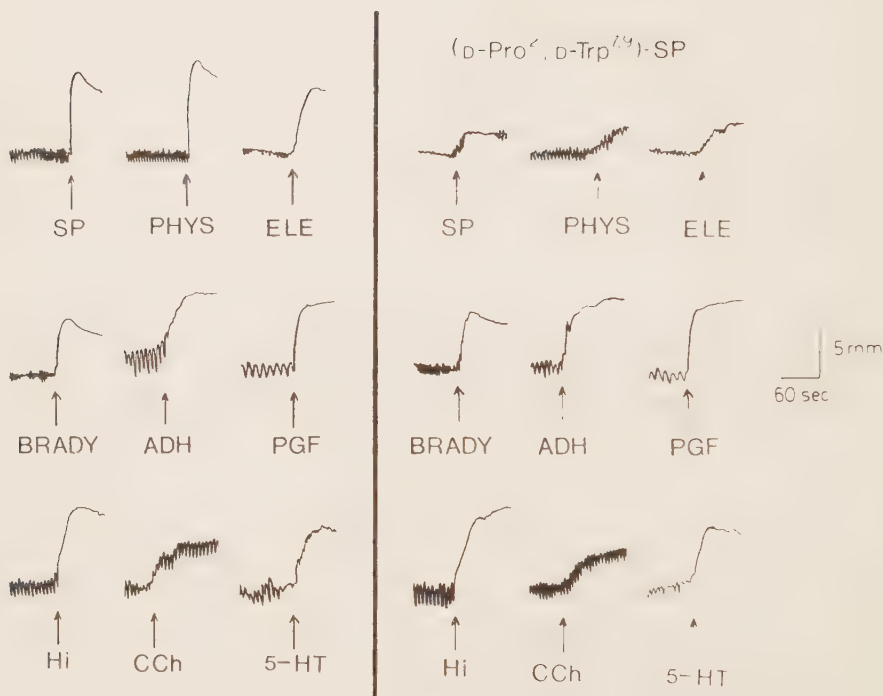


Fig. 5. Contractile response of the isolated guinea pig taenia coli to physalaemin (PHYS), eledoisin (ELE), at a concentration of 10^{-9} M, SP bradykinin (BRADY), histamine (Hi), carbachol (CCh) and serotonin (5-HT) at a concentration of 10^{-8} M, Lys⁸-vasopressin (antidiuretic hormone, ADH) and prostaglandin F_{2α} (PGF) at a concentration of 10^{-7} M, with (right) or without (left) 10^{-5} M $(D\text{-Pro}^2, D\text{-Trp}^{7,9})\text{-SP}$ in the bath. The contractile effect of the SP analogue was allowed to subside before the other compounds were added, usually 5 min later.

the dose response curve for SP was shifted to the right, the pA_2 being 6.1. The estimated slope of the Schild plot (Tallarida, 1979) was -1.0.

Electrical stimulation (1-5 Hz, 3 s) of the taenia elicited a strong contraction. This response was not affected by addition of ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP. Following addition of atropine (10^{-6} M) and guanethidine (5×10^{-6} M), the taenia responded to low-frequency stimulation (1 Hz, 3 s) by relaxing. With higher-frequency stimulation (3-5 Hz, 3 s), the initial relaxation was followed by a contraction on cessation of stimulation. Both these responses were inhibited by TTX. Although the relaxation was unaffected, the amplitude of the contraction was greatly reduced by pretreatment with ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP. At a concentration of 10^{-4} M ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP reduced the contraction by more than 90%.

The sphincter pupillae muscle responded to both SP and electrical stimulation with a slow contraction, in the latter case reaching a maximum 30-40 s after cessation of stimulation. The electrically induced contraction lasted for 150 s. Cholinergic and adrenergic blockade had very little effect on the response to electrical stimulation, but ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP (10^{-5} M) caused a dramatic reduction in the contractile response. As in the taenia coli, the response of the sphincter pupillae muscle to exogenous SP, physalaemin and eledoisin (but not that to carbachol) was greatly inhibited by pretreatment with ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP.

The guinea pig urinary bladder responded to both SP and electrical stimulation with a contraction; the latter response could be completely abolished by TTX. Cholinergic and adrenergic blockade had little or no effect on the response to electrical stimulation. Also ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP failed to inhibit the response.

Analysis of the smooth muscle contraction produced by ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP (VII, VIII). The contractile responses produced by ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP were reduced upon repeated administration and were completely abolished by the H_1 -receptor antagonist mepyramine or pretreatment with the mast cell degranulating compound 48/80.

($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP and Arg-Gln- $\underline{D}$ -Trp-Phe- $\underline{D}$ -Trp-Leu-Met-NH₂ were fairly potent in releasing histamine, much more so than SP. Hence, the findings support the assumption (Paper VII) that the SP analogues cause smooth muscle contraction at least partly through release of histamine from local mast cells.

Role of SP in ocular inflammation (IV, V). Bradykinin, a putative inflammatory mediator (Eisen, 1970; Rocha e Silva, 1970; Erdös, 1979), produced a slow contraction of the isolated sphincter pupillae muscle. The response was reduced upon repeated administration of bradykinin (10^{-8} - 10^{-6} M). After the sphincter pupillae muscle had been made insensitive to bradykinin it retained its capacity to respond to SP. The contractile response to bradykinin, but not that to SP, was abolished by pretreatment with TTX. The contractile responses to SP and bradykinin were greatly reduced or abolished by pretreatment with either of the two SP antagonists (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP (10^{-5} M) and Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH₂ (10^{-5} M). The sphincter pupillae muscle responded to electrical stimulation with a slow contraction, reaching a maximum 30-40 s after cessation of stimulation. Atropine (10^{-6} M) reduced the contractile response to low frequency (5-10 Hz) stimulation by about 25%; responses to high frequency (10-20 Hz) stimulation were not much affected (Paper IV). The atropine-resistant contraction could be abolished by either of the two SP antagonists. As shown in paper VI bradykinin and SP act on separate receptors and (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP does not block the bradykinin receptor. Pretreatment with bradykinin, but not with SP, lowered the contractile response to electrical stimulation. This was a dose-dependent effect, the maximal reduction was approx. 50% and the maximally effective concentration of bradykinin was 10^{-8} M.

Ocular inflammation is associated with an aqueous flare response (AFR) and constriction of the pupil (miosis). Both these responses were evoked by intravitreal injection of bradykinin or SP. Repeated administration of bradykinin 3 or 5 hours later evoked a much reduced response. In contrast, the response to repeated administration of SP was unchanged. Topical application of atropine 1 h before injection of bradykinin did not prevent either the miotic response or the AFR, whereas intravitreal injection of TTX completely abolished the response to bradykinin without affecting the response to SP. In itself, topical application of the two SP antagonists had no effects on the eye but the inflammatory response to the subsequent injection of bradykinin (or SP) was greatly suppressed.

Discussion

Topography of peptide-containing nerve fibers

The gastrointestinal tract is richly innervated. Not long ago it was commonly held that these nerves were either cholinergic or adrenergic despite the fact that both ultrastructural and functional studies had indicated the existence of non-cholinergic and non-adrenergic neuronal elements in the gut (Baumgarten et al., 1970; Burnstock, 1969; 1972; Cook and Burnstock, 1976). Burnstock and associates have argued for quite some time that at least a proportion of these neurones employ ATP and related derivatives as transmitters (Burnstock et al., 1964; Burnstock, 1972, 1979). The more recent immunocytochemical demonstration of several biologically active peptides in gut neurones has offered alternative tentative interpretations of non-cholinergic, non-adrenergic neuronal mechanisms (Hökfelt et al., 1980; Schultzberg et al., 1980; Furness et al., 1981; Håkanson et al., 1981; Håkanson et al., 1982).

The neuropeptides demonstrated have a wide-spread distribution all along the gut: substance P (SP), vasoactive intestinal polypeptide (VIP), enkephalin, somatostatin and GRP. Although the different types of peptide-containing nerve-fibers often are found accumulated within e.g. the myenteric and submucosal plexuses, their over-all topographic distribution and relative frequency differ in a characteristic manner (Sundler et al., 1980) (Fig. 3). The fact that all hitherto described gut neuropeptides occur in the myenteric plexus and/or in the smooth muscle suggests a role for all of them in the regulation of gut motility. The likely targets for the different types of peptidergic nerve fibers based on observations of their distribution within the gut wall are summarized in table 4.

The immunocytochemical findings of neuronal CCK, GRP and neurotensin in the gut of rats and guinea-pig (Polak and Bloom, 1978; Dockray et al., 1979; Larsson and Rehfeld, 1979; Schultzberg et al., 1980; Yanaihara et al., 1981), have been verified immunochemically (see also Carraway and Leeman, 1976a, b; Rehfeld, 1978; McDonald et al., 1979; Hutchison and Dockray, 1981; Yanaihara et al., 1981). With regard to CCK there is immunochemical evidence that most of the immunoreactive material represents CCK-8 (Larsson and Rehfeld, 1979; Dockray et al. 1981). There is, in addition, some evidence that CCK-4 occurs in neuronal elements in the gut (Larsson and Rehfeld, 1979; Rehfeld, 1981).

Table 4

Potential Targets for Neuropeptides in the Mammalian Gut

<u>Substance P</u>	<u>CCK</u>
Neurons (myenteric plexus)	Neurons (myenteric and submucosal plexus)
Smooth muscle	Smooth muscle
Blood vessels	Glands and surface epithelium
<u>VIP</u>	<u>Neurotensin</u>
Neurons (myenteric and submucosal plexus)	Neurons (myenteric plexus)
Smooth muscle (especially sphincters)	Smooth muscle
Glands and surface epithelium	<u>GRP</u>
Blood vessels	Neurons (myenteric and submucosal plexus)
<u>Somatostatin</u>	Smooth muscle
Neurons (myenteric and submucosal plexus)	Glands and surface epithelium
Smooth muscle	<u>β-Endorphin</u>
Glands and surface epithelium	Neurons (myenteric and submucosal plexus)
<u>Enkephalin</u>	Smooth muscle
Neurons (myenteric plexus)	
Smooth muscle	

Origin of peptide-containing nerves in the gut

The gut receives an extrinsic nerve supply from both the parasympathetic (vagus and the sacral nerves) and sympathetic (sympathetic chain via the coeliac and mesenteric ganglia systems). Peptide-containing neuronal elements have been demonstrated in all these structures (Gamse et al., 1979; Hökfelt et al., 1980; Lundberg et al., 1978; Malmfors et al., 1981). In addition the gut has a well developed intrinsic nerve supply emanating from the neurones of the myenteric and submucosal plexuses. The adrenergic fibers, being distributed in all layers of the gut wall, are with a few exceptions extrinsic in origin (Furness and Costa, 1974). Autotransplantation of intestinal segments in the pig did not overtly alter the peptidergic innervation (Malmfors et al., 1981). There is thus good evidence that the majority of the peptide-containing fibers are intrinsic to the gut. However, since extrinsic nerves such as the vagus are rich in peptide-containing axons (Lundberg et al., 1978; Malmfors et al., 1981), we have to assume also the existence of peptide-containing fibers with an extrinsic origin. The exact destination of these extrinsic nerve fibers is unknown; there is evidence that some may terminate in the submucosal plexus and on intramural blood vessels (Costa et al., 1981).

Projections of peptide-containing neurones in the gut

The intramural nervous system of the digestive tract provides an efficient arrangement for the local control of absorptive, digestive and propulsive functions. The effectiveness of such a control mechanism is based on the existence of short reflex arcs (monosynaptic?), mediating immediate and direct responses to a local stimulus, and of longer reflex arcs (polysynaptic?), facilitating an integrated secondary response of more distant regions not directly affected by the local stimulus.

The intramural nervous system of the gut seems designed for such a mode of action. It constitutes an efficient communication system, capable of carrying messages from one end of the gut to the other and also from one layer of the gut wall to another. Some of the nerve fibers may represent dendrites providing the sensory input to the different reflex pathways, but the majority of the fibers are probably efferent. Knowledge of how the intramural neurones project and connect is fundamental to our understanding of how this communication system works. By knowing the polarity and projections of particular peptide-containing nerves, the functional role of each group can be ascertained. Although observations on the mere distribution of peptide-containing neurones within the gut wall have provided valuable information, more conclusive data on their projections have

been obtained using selective denervation experiments. Especially the work of Furness and Costa (c.f. Furness et al., 1981) has gone a long way to reveal some of the mysteries connected with the projections and the polarity of several of the neuronal circuits in the intestine. Lesion experiments have indicated that most SP axons within the plexuses arise from cell bodies within the same or adjacent ganglia and that consequently they project over short distances (around 1 mm) up and down the intestine. SP axons outside the myenteric plexus probably originate from cell bodies in adjacent myenteric ganglia. SP cell bodies occur also in the submucosal plexus; their projections are poorly known although there is evidence that some project to the mucosal layer while others may connect with the myenteric plexus (Jessen et al., 1980). VIP has been proposed as the transmitter released by the enteric inhibitory nerves (Furness and Costa, 1979, 1981). Such a role for VIP is compatible with the finding that VIP neurones in the myenteric plexus project in an anal direction, some of the axons entering the circular muscle layer. The shortest of the descending VIP pathways is about 0.25 mm long. The VIP neurones in the submucosal plexus seem to project to the mucosa. Enkephalin neurones project in both oral and anal directions to neighbouring myenteric ganglia and to smooth muscle. The distribution of the enkephalin fibers suggest that they are directly involved in the control of motility but not in the control of other functions such as blood flow or secretion. The somatostatin neurones, finally, are thought to act as interneurones, since the vast majority of the somatostatin fibers are found within the myenteric and submucous ganglia. Lesion studies indicate that the somatostatin fibers project in an anal direction, mainly to other myenteric ganglia, but none project from anal to oral direction in the guinea pig small intestine. Interestingly, a proportion of the somatostatin neurones seem to project on to other somatostatin cell bodies in an anally directed series, each projection averaging 8-12 mm in length. The cell bodies occurring in the submucosal ganglia seem to project mainly to adjacent submucosal ganglia and to the mucosa. The projections and polarities of the GRP, β -endorphin and neurotensin neurones are unknown.

Ultrastructural localization of neuropeptides in the gut

Application of the immunoperoxidase technique at the ultrastructural level has made it possible to identify the subcellular storage site of three different neuropeptides in the gut: VIP, SP and enkephalin (Håkanson et al., 1981; Larsson, 1977; Sundler et al., 1980). Available data indicate that the neuropeptides reside in large granular vesicles (LGV) of varying electron density and measuring around 1000 Å in diameter. In many fibers LGV

are numerous. Such fibers correspond to the "p-type" fibers first described by Baumgarten et al. (1970) and suggested to be peptidergic. It is not inconceivable that all LGV represent storage sites for neuropeptides and that the presence of LGV in a nerve fiber indicates the presence of a neuropeptide. Since virtually all nerve fibers in the gut - and in other peripheral organs as well - contain at least a few LGV it may be speculated that all autonomic nerves contain neuropeptides in varying amounts and that the number of LGV is a measure of the amount of peptide present.

Are the neuropeptides transmitters?

In a recent review Orrego (1979) listed three major criteria to be fulfilled for a substance to be regarded as a neurotransmitter: 1. Neurovesicular location, 2. Induced release, 3. Identity of action. 1. The transmitter candidate must be shown to exist within neurovesicles. Enzymes capable of synthesizing the transmitter candidate should also occur in the neurone. 2. When nerve fibers are electrically (or otherwise) stimulated, they should release the transmitter candidate. The release should be proportionate to the magnitude of stimulation. The release process must show a true dependence on extracellular calcium concentration and be sensitive to TTX. 3. When a transmitter candidate is applied exogenously it should produce the same postsynaptic effects as the natural transmitter.* Also, the postsynaptic effect of the putative transmitter must be modified by drugs e.g. specific receptor antagonists in the same manner as that of the natural transmitter. Although a neurovesicular localization of the peptides has been established in a few cases only, it appears quite likely that all neuropeptides will eventually prove to have such a localization. There is also much evidence that neuropeptides are released upon nerve stimulation (Fahrenkrug et al., 1978; Gazelius et al., 1981). The bioactivity profiles of the neuropeptides are quite compatible with transmitter roles. The final proof awaits the development of specific receptor antagonists which can be shown to inhibit not only the effects of the peptides but also the response to stimulation of non-cholinergic, non-adrenergic nerves. Such specific antagonists are not available except with respect to the opiate receptors and the SP-receptors.

* As Werman (1966) pointed out, however, the applied transmitter candidate may act by releasing the natural transmitter. The observed effects should then not be the direct effects of the transmitter candidate. This pitfall can be circumvented by applying the test substance under conditions where transmitter release is prevented (e.g. by TTX), provided such a procedure does not modify postsynaptic responses.

Motor effects of the neuropeptides

Many neuropeptides have pronounced effects on gut smooth muscle, and the isolated taenia coli is a convenient model system for evaluating their physiological roles. From the results of such experiments, neuropeptides may be classified in those that act directly on smooth muscle receptors (1) and those that act on neuronal receptors (2) (Fig.6). It should be pointed out that the classification would probably be different with another model system (Holzer, 1982; Fujisawa and Ito, 1982).

1) SP, GRP, CCK-8, neurotensin and VIP produce effects on the isolated taenia that are unaffected by TTX. They therefore act directly on smooth muscle receptors. SP, GRP, CCK-8 and neurotensin contract, while VIP relaxes the isolated guinea-pig taenia coli. Interestingly, neurotensin relaxes the rat ileum and guinea pig colon and contracts the guinea pig ileum and taenia coli (Kitabgi and Freychet, 1978; Kitabgi and Vincent, 1981). The neurotensin-induced contraction of the guinea pig ileum was the result of a nerve-mediated, partly cholinergic process (Kitabgi and Freychet, 1979) whereas neurotensin was found to act directly on the taenia (Kitabgi and Freychet, 1978). Bombesin-like peptides are known to contract smooth muscle in a number of tissues (Erspamer, 1980; Erspamer et al., 1972). Bombesin and probably GRP as well are potent releasers of other neurohormonal peptides (see Erspamer, 1980; Ghatei et al., 1982), and Rökæus and McDonald (1980) observed release of neurotensin-like material from the intestine following administration of bombesin. Such a mechanism does not seem to apply in the isolated taenia coli, since the contractile responses to both GRP and bombesin were TTX-resistant. The contractions produced by CCK-8 can be partly blocked by TTX, suggesting that to a minor extent CCK-8 acts directly on smooth muscle receptors.

2) SP, enkephalins, CCK-8, β -endorphin and possibly somatostatin act on neuronal receptors to modulate neuronal activity. Of these CCK-8 and SP (both of which produce additional effects directly on smooth muscle receptors) act by enhancing the contractile motor response to electrical field stimulation, i.e. they are excitatory neuromodulators, whereas enkephalin, β -endorphin and somatostatin are inhibitory neuromodulators. In the isolated guinea-pig ileum SP potentiates the contractile response to electrical nerve stimulation (Hedqvist and von Euler, 1975), probably the cholinergic component since the response could be blocked by atropine. In fact, SP was recently shown to release acetylcholine (Yau and Youther, 1982). Gastrins and cholecystokinins release acetylcholine from the myenteric plexus of guinea-pig ileum (Del Tacca et al., 1970; Hedner and Rorsman, 1968; Vizi, 1973; Vizi et al., 1972; Hutchison and Dockray, 1981). Hutchison and Dock-

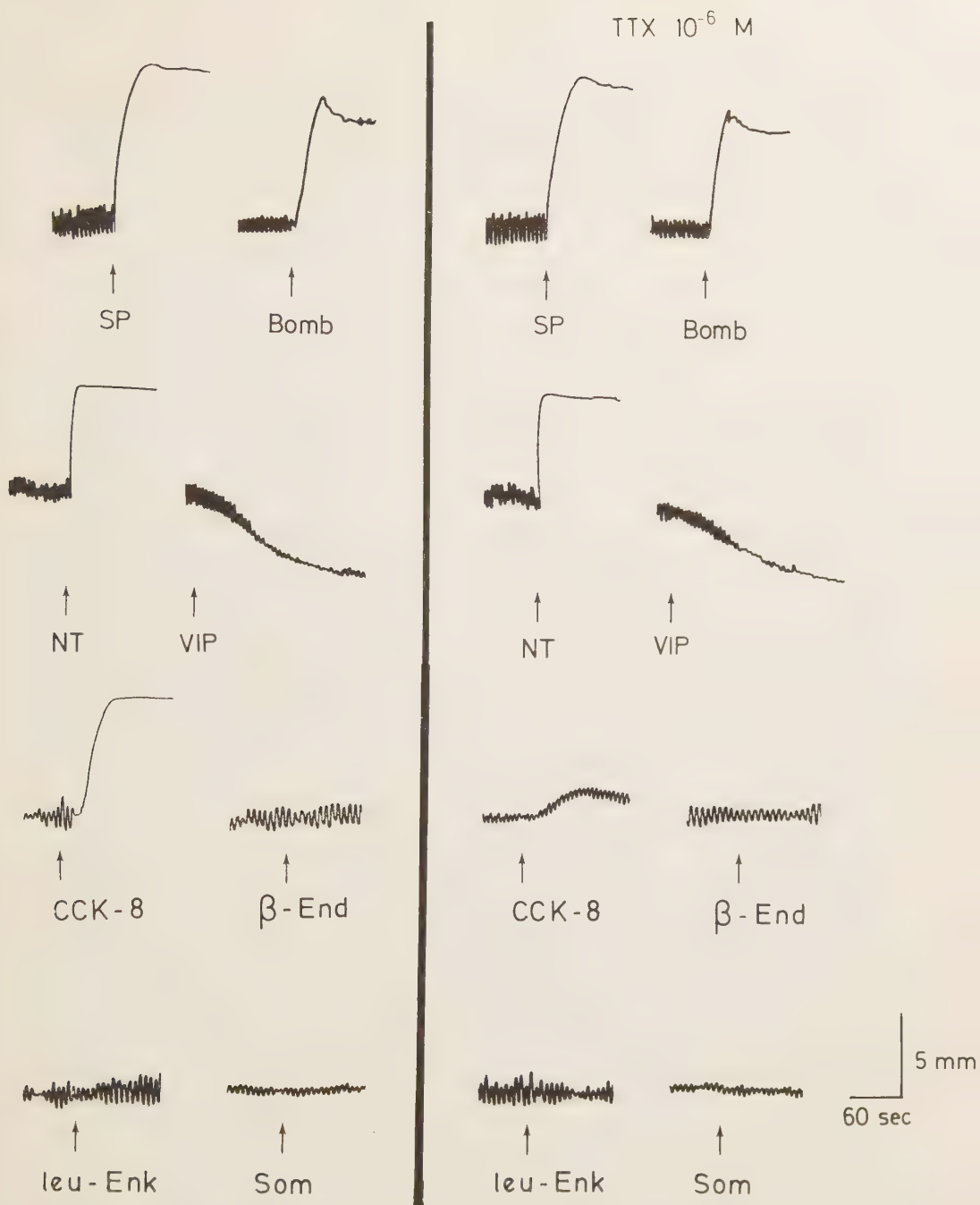


Fig. 6. Effects of neuropeptides on guinea-pig taenia coli in the absence (left) and presence (right) of TTX. Note the reduced contractile response to CCK-8 in the presence of TTX. Substance P (SP); bombesin (bomb); neurotensin (NT); vasoactive intestinal polypeptide (VIP); cholecystokinin octapeptide (CCK-8); β -endorphin (β -end); leu-enkephalin (leu-enk); somatostatin (som).

ray (1981) have suggested that sulphated CCK-8 releases not only acetylcholine but also SP. This is unlikely in the case of the taenia since ($\underline{\text{D}}\text{-Pro}^2$, $\underline{\text{D}}\text{-Trp}^{7,9}$)-SP did not affect the response of the taenia to CCK-8. However, CCK-8 seems to potentiate the contractile response of the taenia to stimulation of SP-ergic nerve fibers. Neither SP nor CCK-8 affected the inhibitory neuronal systems of the gut wall. Enkephalin, and even more so β -endorphin, inhibited the contractile response to stimulation of cholinergic nerves and SP nerves; these effects were reversed by naloxone. β -Endorphin, like many other opiate agonists, effectively prevents the contractions produced by electrical stimulation in a longitudinal muscle strip or intact segment of guinea-pig ileum (Waterfield et al., 1977). Also the contractile response to neurotensin in these preparations is abolished (Monier and Kitabgi, 1981). Neither enkephalin nor β -endorphin affected the inhibitory neuronal systems of the isolated taenia. Somatostatin, finally, has only marginal effects on neuronal responses in the guinea-pig taenia coli. The fact that different types of peptidergic nerve fibers often occur together in small bundles running along the smooth muscle fibers (Schultzberg et al., 1980) may provide a morphological basis for interactions between them. The neuromodulatory effects demonstrated by many of the neuropeptides may well be exerted in such nerve bundles (via axo-axonal contacts) rather than in the intramural plexuses.

Is SP a neurotransmitter?

The SP analogue ($\underline{\text{D}}\text{-Pro}^2$, $\underline{\text{D}}\text{-Trp}^{7,9}$)-SP was shown to block the contractile response to exogenous SP in different smooth muscle systems in vitro. Interestingly, this analogue also blocked the contractile response to SP-related peptides (eledoisin and physalaemin) but not responses to other contractile agents (Fig. 5). These two peptides have been grouped together with SP under the name tachykinins (Erspamer and Melchiorri 1973; Erspamer et al. 1977). It might be suggested that they act on the same receptor, thus explaining why the contractile activity of all three of them was blocked.

As SP antagonists exert some contractile effect of their own it could be argued that the SP blocking properties were due to desensitization of SP receptors instead of SP receptor blockade. Desensitization with SP is a phenomenon known to occur in some tissues (Franco et al. 1975) but could be ruled out as an explanation for the inhibiting effect in this case for the following reasons: 1) With increasing concentrations of the SP antagonist present in the bath the concentration-response curves for SP were shifted to the right. 2) By increasing the concentrations of SP in the

presence of the antagonist the same maximum contractile effect as in control preparations could be obtained. 3) The pA_2 value for ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP was calculated to be 6.1 with a slope of the Schild plot (Tallarida et al. 1979) of -1.0. The theoretical value for the slope of the Schild plot for a competitive antagonist is -1.0. Together these observations suggest that ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP is a specific, competitive SP receptor antagonist.

The contraction produced by the SP antagonist in the taenia was blocked by the histamine H₁-receptor blocking agent mepyramine or by pretreatment with the histamine-releasing mast-cell degranulating compound 48/80. This indicated that the contraction was due to an effect of histamine released from local mast cells. This view was strengthened by the fact that SP analogues were much more potent than SP itself in releasing histamine from isolated peritoneal mast cells. This implies that the design of the clinically useful SP antagonists will require not only improvements in antagonistic potency but also elimination of histamine-releasing activity.

($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP also antagonizes the neuronally mediated non-cholinergic, non-adrenergic contraction of the isolated guinea-pig taenia coli and rabbit sphincter pupillae. These observations together with the immunocytochemical demonstration of SP in nerve fibers in both locations suggests a role for SP as an excitatory motor transmitter. However, one of the primary criteria remains to be fulfilled namely to directly establish a release of SP as a result of neuronal activity. In view of the large variety of peptide-containing nerve fibers in the taenia coli it is surprising that all the contractile responses to field stimulation seem to be blocked by cholinergic and SP-ergic antagonists in combination. It must be realized, however, that electric field stimulation activates all nerve ending simultaneously and that the motor response probably reflects the collective action of a number of excitatory and inhibitory transmitters. A relaxatory response to nerve stimulation might for example mask effects of excitatory transmitters released simultaneously with the inhibitory transmitters.

Although present in nerve fibers in the circular smooth muscle from cat esophagus no pharmacological indications that SP is a motor transmitter in this location could be obtained. SP enhanced responses to field stimulation (Hedqvist and von Euler 1975) in a manner suggesting an additional type of action apart from the one found in the taenia or sphincter pupillae. In a recent paper it was described (Yau and Yother, 1982) that SP increased the release of labelled acetylcholine from strips of guinea-pig small

intestine. This release was blocked by both TTX and a specific SP antagonist indicating that SP in this case acted on SP receptors located on neurones in the preparation.

SP is probably not a motor neurotransmitter in the guinea-pig urinary bladder since although the contractile effect of exogenous SP was reduced the contractile neuronal responses were not affected by the SP antagonist.

Are VIP, enkephalin, somatostatin, GRP, β -endorphin, neurotensin and CCK neurotransmitters?

VIP was found to relax the taenia and has been proposed as a relaxatory neuro-transmitter (Furness and Costa, 1979, 1981; Fahrenkrug et al., 1981). The lack of specific receptor blocking agents for VIP makes it difficult to establish a neurotransmitter role for VIP. The enkephalins had only moderate effects on motor responses whereas β -endorphin strongly inhibited excitatory neuronal responses in the taenia in a naloxone sensitive manner. It might be suggested that β -endorphin acts as an inhibitory agent via presynaptic regulation of transmitter release (Waterfield et al., 1977; Vizi et al., 1977).

Somatostatin had only marginal effects on the motor activity of the taenia and its role in the regulation of smooth muscle activity remains enigmatic. Both GRP and neurotensin contracted the taenia by a direct action on the smooth muscle. A correct assessment of their possible roles as transmitters is hampered by lack of specific blocking agents. CCK-8 stimulated the taenia in quite a complex manner. The resting tension was raised by a partly TTX sensitive mechanism. Atropine also reduced the CCK-8 induced contraction but not as effectively as TTX, indicating that acetylcholine is not the only transmitter released by CCK-8. SP antagonists were without effect. CCK-8 also potentiated responses induced by electrical stimulation but only in the absence of SP antagonists. These results indicate CCK-8 as an indirectly as well as directly acting excitatory neurotransmitter.

SP and ocular inflammation (IV, V, VI)

SP has been proposed to be an inflammatory mediator (Lembeck and Holzer, 1979), for instance in ocular inflammation (Holmdahl et al., 1981). Intra-ocular injection of SP causes vasodilation, breakdown of the blood-aqueous barrier and a rise in intraocular pressure (Bill et al., 1979). SP is also a potent miotic agent (Mandahl and Bill 1980; Masuda et al. 1980; Stjernschantz et al. 1981). Like SP, bradykinin is known to cause inflammation in the eye (Cole and Unger 1974; Eakins 1977), characterized by miosis, hyperemia, and breakdown of the blood-aqueous barrier (aqueous flare res-

ponse, AFR), thereby mimicking the response to trauma. SP and bradykinin act on separate receptors (paper VI). As both the in vivo response, AFR and miosis, and the in vitro response, contraction of the sphincter pupillae muscle, to bradykinin could be blocked by either TTX or SP antagonists it was suggested that bradykinin acts by releasing neuronal SP. Bradykinin seems to act on a neuronal pool of SP that only partly corresponds to that which can be released by electrical stimulation as the sphincter still responded to such stimulation when the preparation had become unresponsive to bradykinin. Similarly, the results of the studies on the bradykinin-evoked inflammatory response in the rabbit eye indicate that the symptoms are caused by neuronal SP released by bradykinin.

The inflammatory response in the eye is thought to depend upon agents released from peripheral sensory nerve fibres (Butler & Hammon, 1977; Holmdahl et al., 1981). SP is a likely candidate for several reasons: 1) Exogenous SP evokes symptoms of inflammation (Bill et al., 1979; Butler & Hammond, 1980; Holmdahl et al., 1981); 2) Capsaicin (an SP-releasing agent) evokes inflammation (Camras & Bito, 1980; Bynke, 1982); 3) SP is a constituent of sensory nerve fibers (Otsuka & Konishi, 1977), probably including those of the trigeminal nerve (paper IV); 4) Sectioning of the trigeminal nerve results in suppression of inflammation (Butler & Hammond, 1980; Butler et al., 1981); 5) Specific antagonists to SP suppress inflammation (Holmdahl et al., 1981).

The present results support the view that neuronal SP plays a key role in the inflammatory process.

Summary

The occurrence and topographical distribution of various neuropeptide containing nerve fibers in the guinea-pig intestine, rabbit eye and cat, guinea-pig and pig esophagus is described. The guinea-pig intestine was found to harbour nerve fibers containing immunoreactive substance P (SP), vasoactive intestinal polypeptide (VIP), enkephalin, somatostatin, cholecystokinin (CCK), β -endorphin, neurotensin and gastrin releasing peptide (GRP). Cell bodies containing SP, VIP, enkephalin, CCK, β -endorphin and GRP could be detected in the myenteric plexus of guinea-pig intestine indicating an intrinsic origin of these nerve fibers. The anterior segment of the rabbit eye contained immunoreactive SP fibers in the iris, ciliary body and ciliary processes. In addition, blood vessels in the uvea were often surrounded by SP fibers. SP-immunoreactive nerve fibers were fairly numerous in the lower esophagus of the cat and guinea-pig but few in the pig. Only in the cat were immunoreactive cell bodies detected in the esophageal wall. By immunostaining ultrathin sections from guinea-pig intestine it was possible to show that neuronal SP, VIP and enkephalin are stored in large dense cored vesicles.

The smooth muscle preparations tested responded to electrical field stimulation also in the presence of atropine and guanethidine. The responses were in the guinea-pig taenia coli: a relaxation followed by a contraction; in the cat esophageal circular smooth muscle: a relaxation and in the rabbit sphincter pupillae a contraction. These responses were all sensitive to the neuronal blocker tetrodotoxin (TTX). The neuropeptides were found to have pronounced effects on the motor activity of the smooth muscle preparations: SP, CCK, neurotensin and GRP were found to contract whereas VIP gave a relaxation response in the taenia coli. The cat esophageal smooth muscle and the rabbit sphincter pupillae were contracted by SP. β -Endorphin and enkephalin were found to inhibit all electrically induced contractile responses in the taenia coli and the cholinergic contractile response in the cat esophagus. The non-cholinergic, non-adrenergic contractile responses in the taenia coli and in the sphincter pupillae as well as the contractile response to exogenous SP were inhibited by specific SP antagonists. These observations together with the neurovesicular localization of SP suggest that SP acts as an excitatory neurotransmitter in these preparations.

Bradykinin contracts the isolated sphincter pupillae and causes ocular inflammatorion in the rabbit. These responses were sensitive to both TTX and specific SP antagonists indicating that bradykinin acts by releasing neuronal SP and that SP is likely to play an important role in inflammatory processes in the eye.

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Nerves Containing Substance P, Vasoactive Intestinal Polypeptide, Enkephalin or Somatostatin in the Guinea-Pig Taenia Coli

Distribution, Ultrastructure and Possible Functions

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Summary. The guinea-pig taenia coli is rich in peptide-containing nerves. Nerve fibres containing substance P (SP), vasoactive intestinal peptide (VIP), or enkephalin, were numerous in the smooth muscle while somatostatin fibres were very few. Nerve fibres displaying SP or VIP immunoreactivity were numerous in the myenteric plexus. Enkephalin nerve fibres were fairly numerous in the plexus while somatostatin nerve fibres were sparse. Nerve cell bodies containing immunoreactive SP or VIP were regularly seen in the plexus. Delicate varicose elements of the different types of nerve fibres were found to ramify around nerve cell bodies in a manner suggestive of innervation.

In the electron microscope the various peptide-storing nerve fibres (i.e., elements containing SP, VIP or enkephalin) were found to contain a varying number of fairly large, electron-opaque vesicles in the varicose swellings. These vesicles represent the storage site of the neuropeptides.

The isolated taenia coli responded to electrical nerve stimulation with a contraction. After cholinergic and adrenergic blockade the contractile response was replaced by a relaxation followed by a contraction upon cessation of stimulation. SP contracted the taenia while VIP caused a relaxation. The enkephalins raised the resting tension slightly while somatostatin had no effect. These observations are compatible with a role for SP as an excitatory neurotransmitter and for VIP as an inhibitory one, and with the view that both SP neurones and VIP neurones act as motor neurones. In preparations contracted by SP the electrically induced contractions were reduced in amplitude while the electrically induced relaxations seen after adrenergic and cholinergic blockade were enhanced in amplitude. In preparations relaxed by VIP there was an increased contractile response to electrical stimulation, while in the atropine + guanethidine-treated preparation the electrically induced

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relaxations were reduced in amplitude. The enkephalins reduced the contractile response to electrical stimulation, while somatostatin induced a very small reduction in the amplitude of such responses. These observations suggest that SP neurones and VIP neurones may play additional roles as interneurones. Somatostatin neurones probably act as interneurones. Enkephalin-containing fibres may serve to modify the release of transmitter from other nerves in the smooth muscle, perhaps through axo-axonal arrangements. Alternatively, the enkephalin nerve fibres in the smooth muscle are afferent elements involved in mediating sensory impulses to the myenteric plexus.

Key words: Peptidergic nerves – Guinea-pig taenia coli – Substance P – Vasoactive intestinal peptide – Somatostatin – Enkephalin – Ultrastructure – Histochemistry.

The many functions of the digestive tract are controlled by a complex interplay of hormonal and neuronal signals. The nerve fibres in the gut are either extrinsic or intrinsic in origin. The extrinsic nerve fibres are derived from the parasympathetic nervous system via the vagi and the sacral nerves and from the sympathetic nervous system via the prevertebral ganglia (coeliac, superior mesenteric and inferior mesenteric). The intrinsic nerve fibres have their cell bodies in the myenteric and the submucosal plexuses. The intrinsic innervation of the gut probably functions as a "transducer" to signals from the extrinsic nerves, thus constituting an intermediary or final link in the neuronal chain connecting the central nervous system with the target cells. In addition, the high degree of autonomy displayed by the gut implies that the intrinsic nerves also play a more independent role. They are probably active in eliciting local reflexes in response to for instance chemical stimuli and distension and they have the inherent capacity to take control when the extrinsic nerves are cut. Although cholinergic neurones probably represent a considerable proportion of the intrinsic neurones of the gut wall, many neurones in the plexuses seem to be non-cholinergic (and non-adrenergic). They have been referred to as p-type because they ultrastructurally resemble the peptide-containing neurosecretory nerves in the posterior pituitary (Baumgarten et al. 1970; see also Cook and Burnstock 1976). These p-type nerves seem to contain peptides, such as substance P (SP), vasoactive intestinal peptide (VIP), somatostatin or enkephalin (cf. Hakanson et al. 1980; Sundler et al. 1980). All these neuropeptides have strong effects on the gut (see Grossman 1974; Konturek 1976, 1978; Bury and Mashford 1977; Fahrenkrug 1979). Other putative neuropeptides have been demonstrated in the gut wall (see Furness and Costa 1980; Schultzberg et al. 1980; Sundler et al. 1980). One major issue at present is whether the neuropeptides are released upon nerve stimulation to act as transmitters.

Morphological observations suggest that the myenteric and submucosal plexuses are supplied with a large number of peptide-containing interneurones (cf. Jessen et al. 1980), which may act to modify the function of other neurones. In this way outgoing signals from the plexus are the result of filtering an incoming impulse flow through a closely knit neuronal web.

The myenteric plexus is probably concerned predominantly with the regulation of smooth muscle activity. The mechanical activity of smooth muscle lends itself to easy registration and it was therefore decided to use the taenia coli of the guinea pig

as an experimental model in an attempt to find tentative answers to the following questions: 1. What are the components of the myenteric plexus? Which types of neurones affect the smooth muscle cells directly (motor neurones) and which are interneurones and/or sensory neurones? 2. Do the neuropeptides act as transmitters? 3. What are the effects of the neuropeptides on the spontaneous and neurally evoked mechanical activity of the taenia coli? 4. Which responses of the smooth muscle are evoked by stimulation of neurones in the myenteric plexus and which neurotransmitters are responsible? - We have tried to find tentative answers to these questions by a combined morphological and mechanical study. The present study is concerned with SP, VIP, somatostatin, met-enkephalin and leu-enkephalin.

Materials and Methods

Tissue Preparation

Investigations were performed on the isolated taenia coli preparations of male guinea pigs, weighing 300–500 g. The animals were stunned by a blow on the head and bled to death. Taenia strip preparations, consisting of longitudinal smooth muscle and attached myenteric plexus, were dissected from the caecum as described by Burnstock et al. (1966) and placed in Krebs' solution (for composition see below) at 4°C for about 1 h before start of motor activity studies.

Histochemistry

Fresh tissue specimens were frozen in a propane-propylene mixture at the temperature of liquid nitrogen and freeze-dried. The specimens were then vapour-fixed with either diethylpyrocarbonate or formaldehyde and embedded in paraffin. Sections were cut at 5 µm. Alternatively the specimens were immersed overnight in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2. After thorough rinsing in buffer containing 5% sucrose the specimens were frozen on dry ice and sectioned in a cryostat at 10–15 µm. Sections from formaldehyde vapour fixed specimens were mounted in Entellan® and examined in a fluorescence microscope for formaldehyde-induced fluorescence as described for indolamines and

Table 1. Characteristics of antisera used

Antisera raised against	Code No.	Working dilution		Source	References
		immuno-fluorescence	PAP staining		
1. Substance P	SP-8	1:160	1:2560	P. Emson, Cambridge University, Cambridge, England	Brodin et al. 1981
2. VIP	5603	1:80	1:5120	J. Fahrenkrug Bispebjerg Hospital Copenhagen, Denmark	Larsson et al. 1976
3. Leu-Enkephalin	170 ^a	1:60	1:240	K.-J. Chang Burroughs Wellcome Research Triangle Park, N.C., USA	Alumets et al. 1978
4. Somatostatin	19578	1:80	1:1280	M.P. Dubois, Institut Nationale de Recherche Agriculture Nouzilly, France	Dubois 1975 Alumets et al. 1977

^a Cross-reacts with met-enkephalin

catecholamines (Björklund et al. 1972). Cryostat sections and sections from diethylpyrocarbonate-fixed specimens were processed for the immunohistochemical demonstration of SP, VIP, somatostatin or enkephalin using previously characterized rabbit antisera (Table 1). It should be noted that although the enkephalin antiserum was raised against leu-enkephalin, it cross-reacts to some extent with met-enkephalin at the immunocytochemical level (Alumets et al. 1978). The site of antigen-antibody reaction was visualized by the indirect immunofluorescence method of Coons et al. (1955) or by the immunoperoxidase (peroxidase-antiperoxidase, PAP) technique of Sternberger (1974). For controls we used antisera which had been inactivated by the addition of excess amounts of antigen (10–100 µg, ml).

Electron Microscopy

Tissue specimens were immersed in fixative consisting of 0.5% glutaraldehyde and 3.5% formaldehyde in 0.075 M phosphate buffer, pH 7.2, for 1 to 4 h. They were postfixed in 1% OsO₄ for 2 h, dehydrated in graded ethanol solutions, contrasted en bloc for 1/2 h in a mixture of 1% phosphotungstic acid and 0.5% uranyl acetate in ethanol and embedded in Epon. Ultrathin sections were exposed to the antisera against SP, VIP, enkephalin or somatostatin. The site of antigen-antibody reaction was revealed by the PAP procedure (see above). For details see Alumets et al. (1977, 1978).

Procedure for Studying the Mechanical Activity of the Taenia Coli

The taenia coli preparation was mounted vertically on a perspex holder in a 10 ml organ bath thermostated at 37°C. One end was attached to a rigid support and the free end to a lever connected via a spring to a Grass FT 03 force displacement transducer or to a photoelectric transducer for isotonical registration of mechanical activity (Ishida and Urakawa 1974). In all experiments the load on the muscle was set at 0.2 g. Platinum ring electrodes were placed on either side of the muscle with a constant electrode distance of 5 mm. The electrodes were connected to a Grass S 4C stimulator for field stimulation with square wave pulses (supramaximal voltage; 0.5–1 msec duration). The muscles were stimulated with trains of pulses lasting for 3–5 s and with a frequency of 1–5 Hz (unless otherwise stated), one train every 2 min. The polarity of the electrodes was shifted after each stimulation period in order to avoid polarisation. The mechanical activity of the preparation was recorded continuously throughout the experiment on a Grass model 7 Polygraph. The bathing fluid was a modified Krebs solution with the following composition (in mM): NaCl 133; NaHCO₃ 16.3; KCl 4.7; MgCl₂ 1.0; NaH₂PO₄ 1.4; CaCl₂ 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7% CO₂ in O₂ giving a pH 7.2–7.3. The preparations were allowed to equilibrate in the bath for 60 min before experimentation started. Concentration-response curves were constructed by adding step-wise increasing amounts of peptides to the bath. Between each concentration the bath was rinsed. With peptides affecting the basal tension the next concentration was not tested until the effect of the preceding one had been washed away.

The following peptides were used: synthetic SP (Beckman, Geneva, Switzerland), pure porcine VIP (a generous gift from Professor V. Mutt at Karolinska Institutet, Stockholm, Sweden), synthetic somatostatin and met- and leu-enkephalin (UCB, Bruxelles, Belgium). The tetrodotoxin used came from Sankyo Co. Ltd., Tokyo, Japan.

Results

Histochemical Observations

Adrenergic nerve fibres and various types of peptide-containing nerve fibres were seen to run in thin nerve bundles and as single fine-varicose terminals along the longitudinal smooth muscle fibres. Of the peptide-containing nerve fibres, those containing immunoreactive SP, VIP or enkephalin were quite numerous (Fig. 1), while those containing somatostatin were very few. Adrenergic nerve fibres were numerous in the myenteric plexus (Fig. 2). In addition, rather many nerve fibres within the plexus displayed SP or VIP immunoreactivity (Fig. 3); enkephalin nerve fibres were fairly numerous while somatostatin fibres were rather few (Fig. 3). Nerve cell bodies containing immunoreactive SP were numerous, while VIP-containing cell bodies were moderate in number. Enkephalin-containing cell bodies



Fig. 1. Immunohistochemical demonstration of nerve fibres containing substance P (a) VIP (b), or enkephalin (c) in guinea-pig taenia coli. The smooth muscle is richly supplied with peptide-containing nerve fibres. $\times 200$



Fig. 2. Formaldehyde-induced fluorescence of adrenergic nerve fibres and cell bodies (*arrow*) in the myenteric plexus of taenia coli. Scattered nerve fibres in the smooth muscle. $\times 350$

were very few and somatostatin-containing cell bodies were not seen at all. Delicate, varicose fibres of all the different types were seen to ramify around nerve cell bodies within the plexus. SP, VIP, enkephalin and somatostatin containing fibres were found also in the internodal strands connecting the ganglia of the myenteric plexus. The observations are summarized in Table 2.

Ultrastructural Observations

Nerve fibres characterized by the presence of large opaque vesicles were numerous in the myenteric plexus (Fig. 4a). Such fibres were regularly seen also in the smooth muscle. By immunostaining ultrathin sections it was possible to show that the various peptides (SP, VIP or enkephalin) were stored exclusively in these large vesicles (Fig. 4b, c). The various types of peptide-containing nerve fibres were ultrastructurally very similar. No immunostaining for somatostatin could be obtained.

Spontaneous and Neurally Evoked Mechanical Activity

When working against the load (0.2 g), the preparation had a fairly regular spontaneous activity. Upon electrical field stimulation the preparations responded with a contraction persisting throughout the stimulation period. With increasing stimulation frequencies the contraction increased in amplitude (Fig. 5). After blockade of the muscarinic cholinergic receptor by atropine sulphate (10^{-6} M) the preparations invariably responded to electrical stimulation with a relaxation (Fig. 6), suggesting that the contraction is mediated by acetylcholine. Blockade of catecholamine release by guanethidine (5×10^{-6} M) did not affect the response to electrical stimulation at a stimulation frequency below 5 Hz. At low stimulation frequencies (below 5 Hz) the relaxation in the presence of atropine + guanethidine

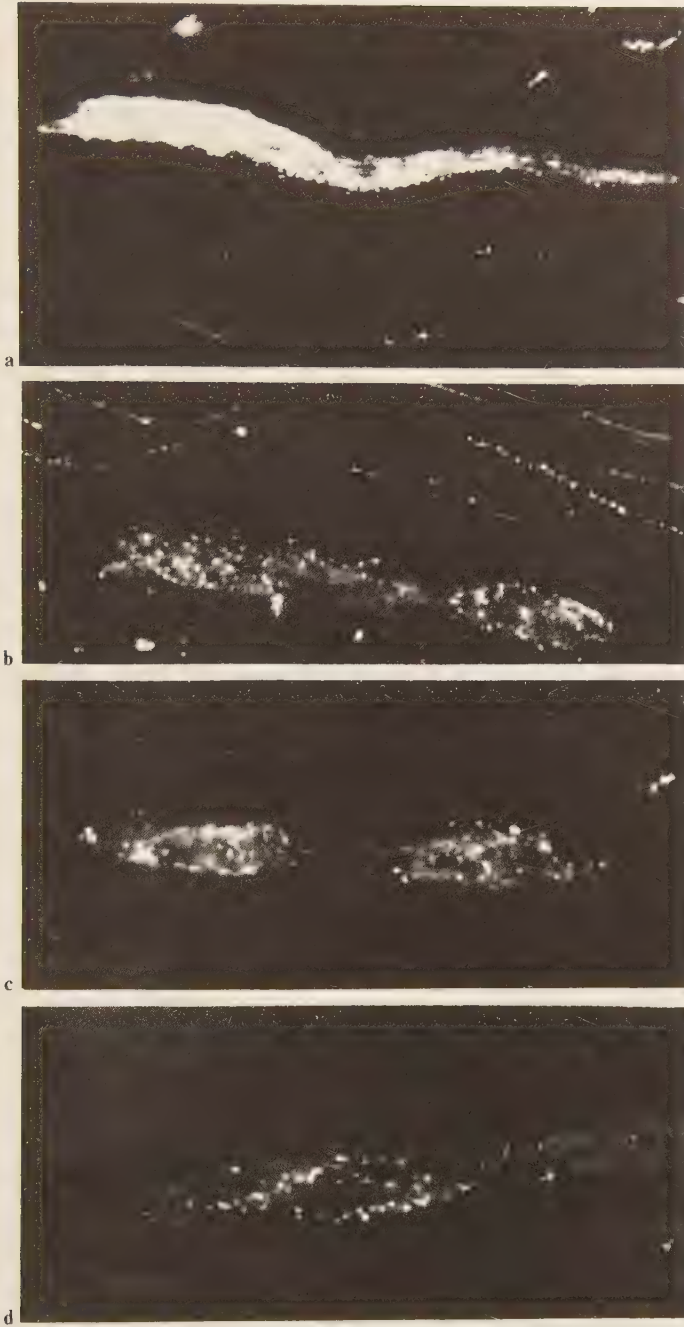


Fig. 3. Immunohistochemical demonstration of peptide-containing neuronal elements in the myenteric plexus of the taenia coli. The sections were stained for: **a** Substance P, **b** VIP, **c** enkephalin, and **d** somatostatin. Substance P fibres are numerous within the plexus which also contains immunoreactive cell bodies. VIP and enkephalin fibres are somewhat less numerous, while those containing somatostatin are relatively few. $\times 300$

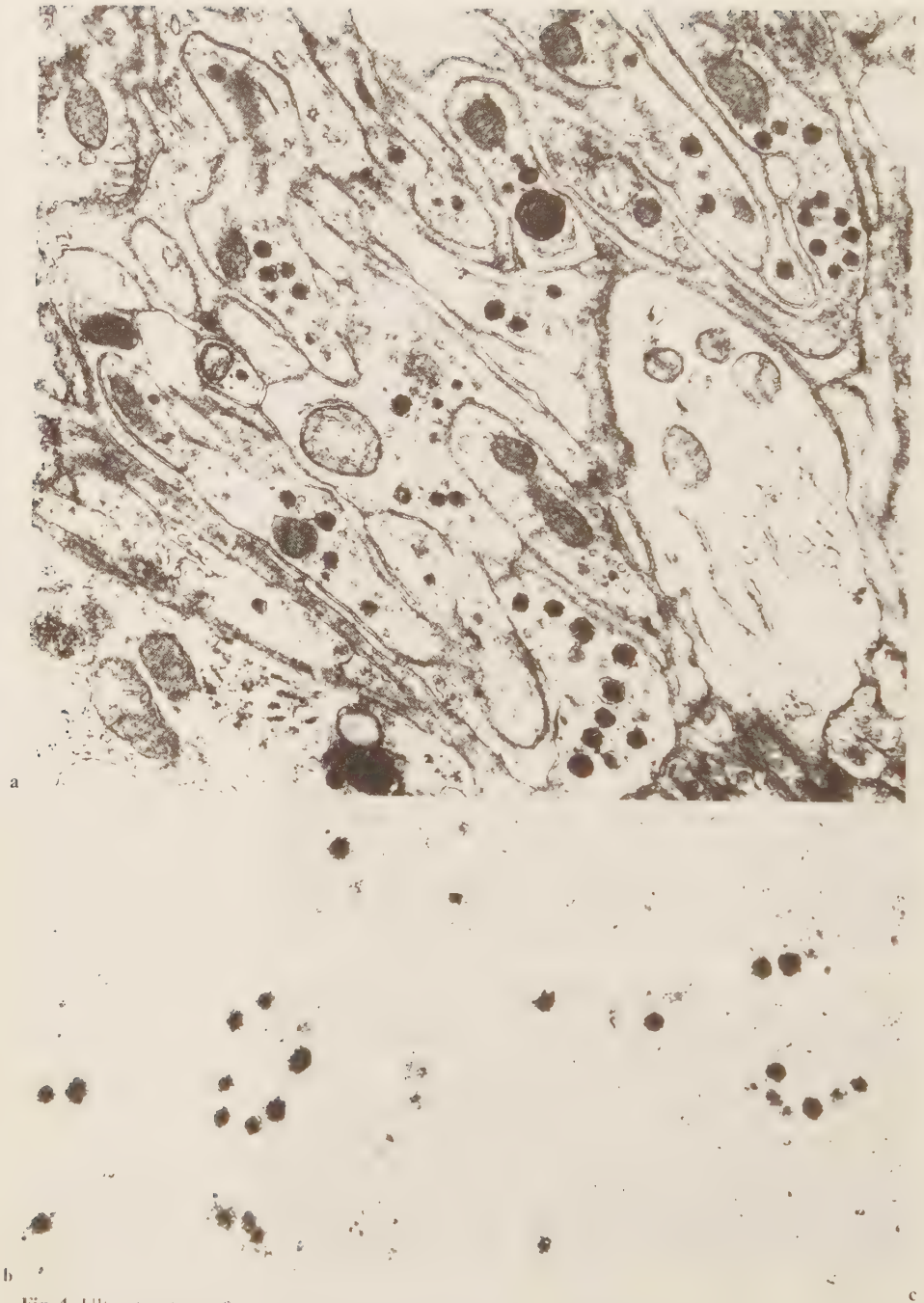


Fig. 4. Ultrastructure of nerve profiles in myenteric plexus. **a** Routinely stained section. Several nerve profiles display features characteristic of p-type nerves in that they contain numerous fairly large electron opaque vesicles. **b** and **c** Sections immunostained (PAP technique) for enkephalin (**b**) and substance P (**c**). Reaction product is accumulated over large neurovesicles exclusively. $\times 25,000$

Table 2. Distribution and relative frequency of peptidergic nerve fibres and cell bodies in the guinea-pig taenia coli

Neuropeptide	Nerve fibers		Nerve Cell Bodies*
	Smooth Muscle	Myenteric Plexus	
substance P	++	+++	+++
VIP	+++	++	+
enkephalin	+++	++	(+)
somatostatin	(+)	+	0

(+) very few; + moderate number; ++ fairly numerous; +++ numerous

* The failure to detect cell bodies does not necessarily mean that they are absent

persisted throughout the stimulation period while at frequencies from 5 Hz and higher the preparations gave a biphasic response: first a relaxation and then a contraction (see also Burnstock 1972).

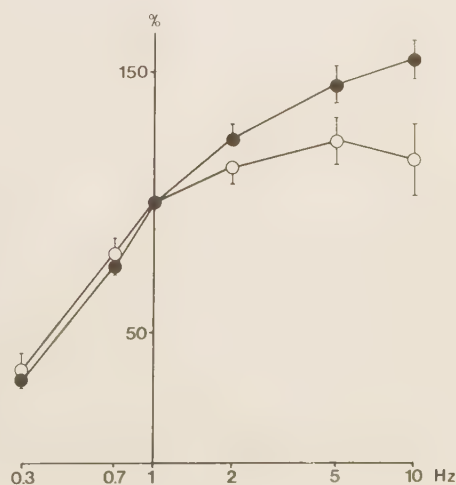
Electrical stimulation in the presence of the opiate receptor antagonist naloxone caused a relaxation followed by a small contraction; this response was obtained in control specimens and after the addition of guanethidine. Naloxone was without effect on atropinized specimens. Following the addition of atropine + guanethidine, the amplitude of the electrically induced relaxation increased in the presence of naloxone (Figs. 6, 7).

Tetrodotoxin (10^{-6} M), a drug known to block nervous conduction specifically (Kao 1966), abolished all responses to electrical stimulation (see Fig. 8).

Effects of Neuropeptides on Resting Tension

SP contracted the taenia in a concentration-dependent manner (Figs. 8a, 9a). This response was not affected by atropine + guanethidine, or by tetrodotoxin (Fig. 8a). VIP relaxed the taenia in a concentration-dependent manner (Figs. 8b, 9b). This

Fig. 5. Effect of different stimulus frequencies on electrically evoked contractions (●) of normal taenia coli and on electrically evoked relaxations (○) following the addition of atropine (10^{-6} M) and guanethidine (5×10^{-6} M). The preparations were stimulated for 3 s every 2 min. Ordinate: Percentage of the amplitude of the response obtained at 1 Hz. Abscissa: Stimulus frequencies (note log scale). Each value is the mean of 28–59 experiments. Vertical bars give SEM. The shaded area indicates the range of frequencies used in subsequent studies



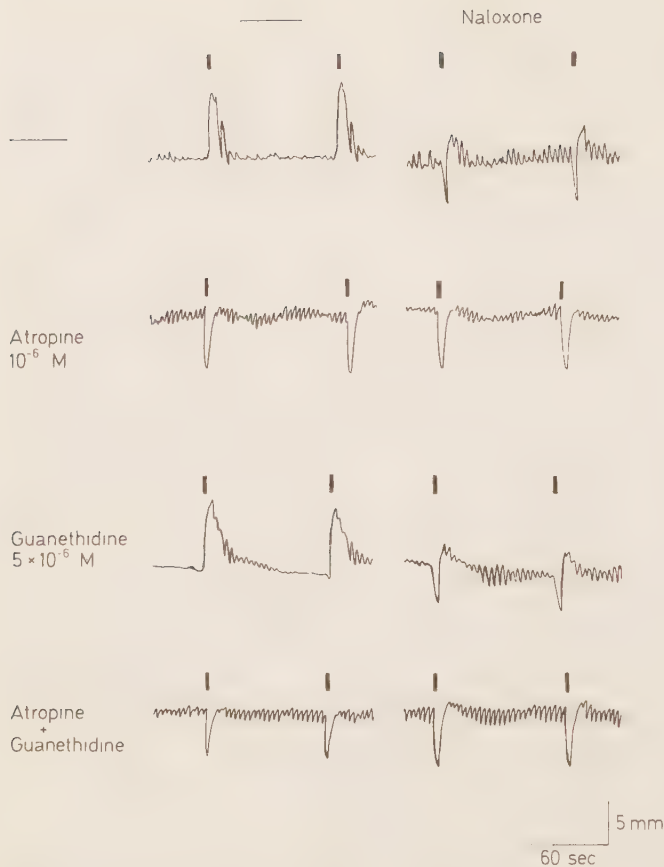


Fig. 6. Typical registration of electrically evoked responses with (right panels) and without naloxone (10^{-4} M) (left panels). Electrical stimulation (1 Hz for 3 s) is indicated. With control taenia and in the presence of guanethidine, naloxone changed the response from contraction to relaxation. In the presence of atropine alone electrically induced relaxations were not affected by naloxone. In the presence of atropine + guanethidine the relaxations were enhanced

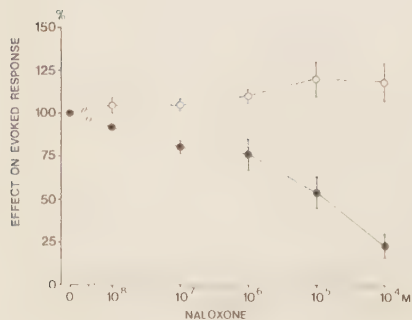


Fig. 7. Effect of naloxone on the electrically evoked contractions (●) of normal taenia coli and on the relaxations (○) following addition of atropine (10^{-6} M) and guanethidine (5×10^{-6} M). The preparations were stimulated with 1 Hz for 3 s every 2 min. Ordinate: Percentage of the amplitude of the response obtained before addition of naloxone. Abscissa: Naloxone concentration (M). Each value is the mean of 4–7 experiments. Vertical bars give S.E.M.

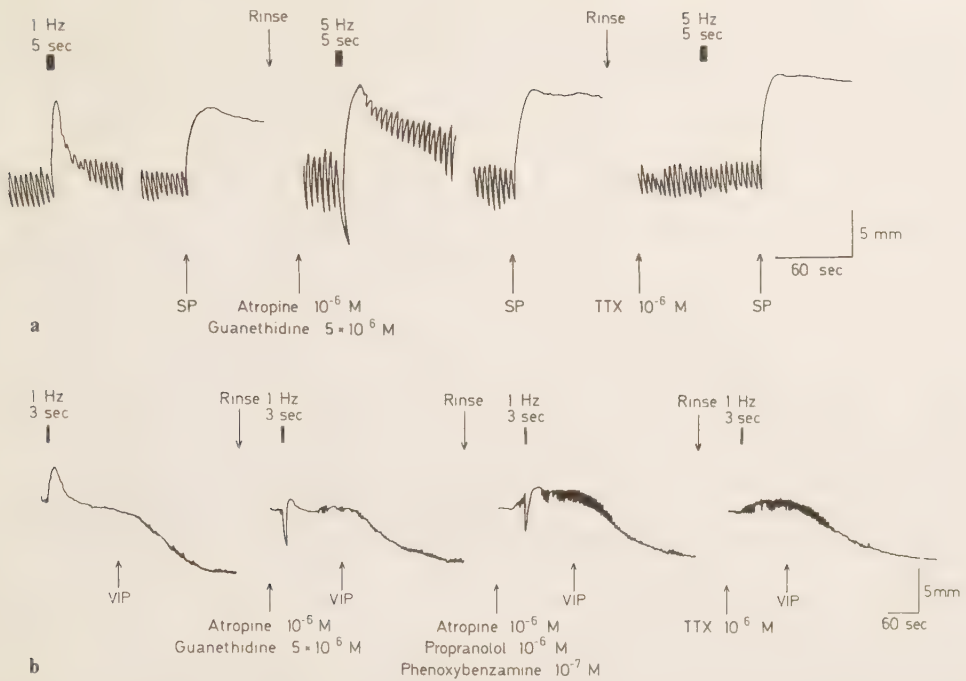


Fig. 8. a Comparison between the response to electrical field stimulation and the response to SP (10^{-8} M). Initially, both electrical stimulation (1 Hz for 5 s) and SP gave contractions. Following cholinergic and adrenergic blockade the response to electrical stimulation (5 Hz for 5 s) was biphasic, while the response to SP was unaffected. Tetrodotoxin abolished all responses to electrical stimulation without affecting the response to SP. Electrical stimulation is indicated. **b** Comparison between the response to electrical field stimulation and the response to VIP (5×10^{-9} M). Initially, electrical stimulation (1 Hz for 3 s) evoked a contraction while VIP gave a relaxation. Following cholinergic and adrenergic blockade electrical stimulation evoked a relaxation while the response to VIP was unaffected. Tetrodotoxin blocked the electrically evoked response without affecting the response to VIP. Electrical stimulation is indicated

response was not influenced by atropine (10^{-6} M) + guanethidine (5×10^{-6} M), by atropine + propranolol (10^{-6} M) + phenoxybenzamine (10^{-7} M), or by tetrodotoxin (10^{-6} M) (Fig. 8b). Met- and leu-enkephalin in concentrations above 10^{-6} M raised the resting tension slightly (Fig. 10). Somatostatin in doses up to 10^{-5} M did not appreciably affect the resting tension (not shown in Figure).

Effects of Neuropeptides on Neurally Evoked Mechanical Activity

The change in resting tension induced by SP and VIP also changed the ability of the preparation to respond to electrical stimulation (Fig. 11). In the SP contracted taenia the electrically induced contractions were reduced in amplitude while the relaxations seen after addition of atropine + guanethidine were enhanced in amplitude. In a preparation relaxed by VIP an increased contractile response to electrical stimulation could be observed. When the atropine + guanethidine-treated muscle was relaxed by addition of VIP to the bath, the electrically induced

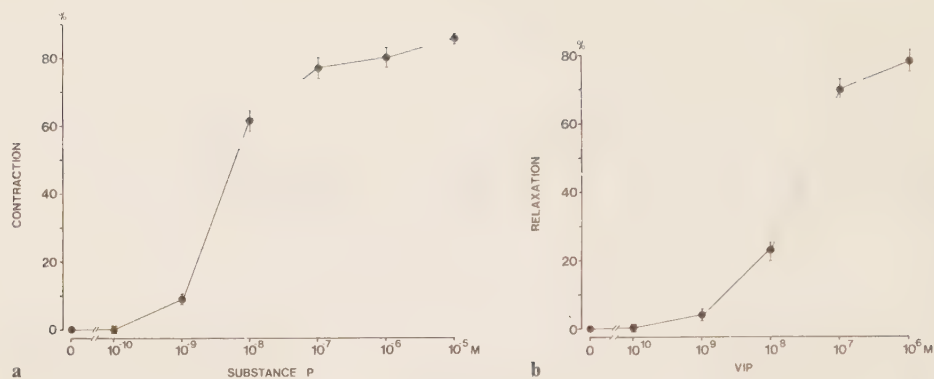


Fig. 9. a Concentration-response curve showing effect of SP on the tension of the guinea pig taenia coli. Ordinate: Increase in tension as a percentage of the contraction induced by carbachol (10^{-5} M). Abscissa: SP concentration (M). Each value is the mean of 4–8 experiments. Vertical bars give S.E.M. **b** Concentration-response curve showing effect of VIP on the tension of the guinea-pig taenia coli. Ordinate: Decrease in tension as a percentage of the relaxation induced by isoproterenol (10^{-5} M). Abscissa: VIP concentration (M). Each value is the mean of 9–15 experiments. Vertical bars give S.E.M.

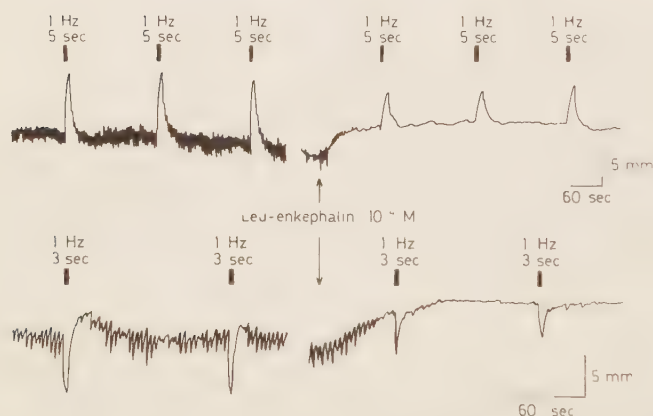


Fig. 10. Registrations showing the effect of leu-enkephalin on the resting tension of the taenia coli and on the amplitude of electrically evoked responses. The top panel shows the activity of a control preparation while the one below shows the activity in the presence of atropine (10^{-6} M) and guanethidine (5×10^{-6} M). Electrical stimulation is indicated.

relaxations were reduced in amplitude (or abolished) (Fig. 11). There was still a rebound contraction however upon cessation of stimulation.

The enkephalins reduced the contractile response to electrical stimulation (Fig. 10 and 12a). This effect could not be reversed by naloxone (in concentrations up to 10^{-4} M). Somatostatin induced a very small reduction in the amplitude of the electrically induced responses in both the normal and the atropine + guanethidine-treated preparations (Fig. 12b).

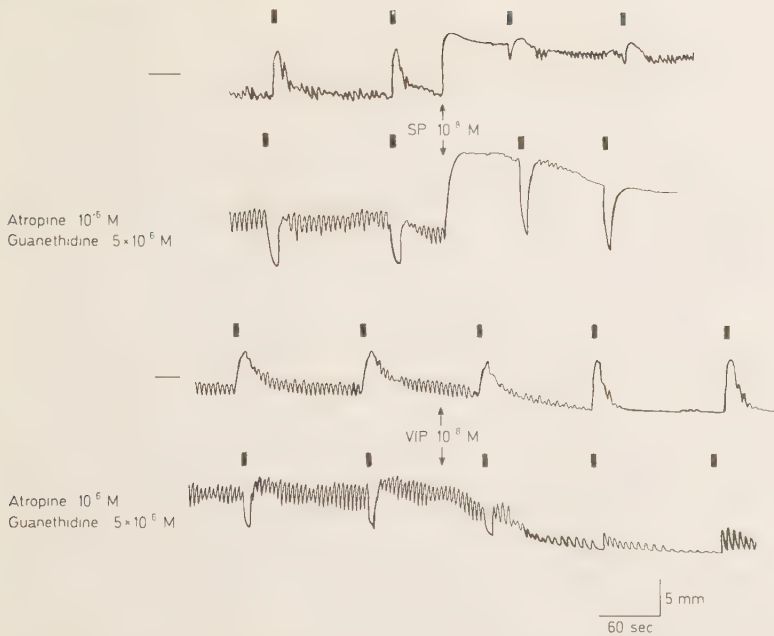


Fig. 11. The effects of SP (panels one and two) and VIP (panels three and four) on the resting tension and on the electrically evoked responses of the taenia coli. SP and VIP were added to the bath when indicated. SP raised and VIP lowered the resting tension. When the muscle was contracted by SP the contractile responses to field stimulation diminished (top panel) and the relaxatory responses increased in amplitude (second panel). In the taenia relaxed by VIP the situation was the opposite. The contractile responses were increased in amplitude (third panel) and the relaxatory responses were reduced in amplitude (fourth panel). Electrical field stimulation (1 Hz for 3 s) is indicated

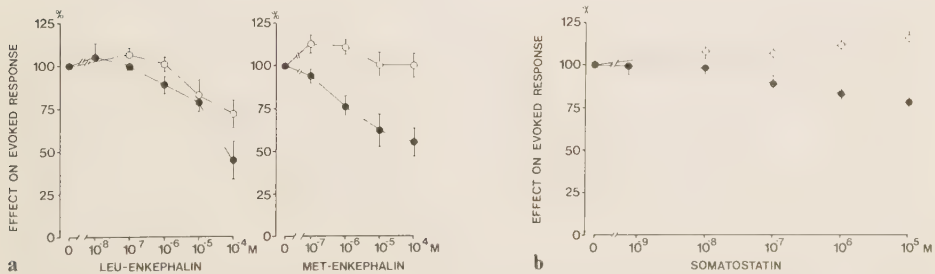


Fig. 12. **a** Concentration-response curves showing the effects of leu-enkephalin (left) and met-enkephalin (right) on electrically evoked contractions (●) in normal taenia coli and relaxations (○) following addition of atropine (10^{-6} M) and guanethidine (5×10^{-6} M). The preparations were stimulated with 1 Hz for 3 s every 2 min. Ordinate: Percentage of the amplitude of the response obtained before addition of enkephalin. Abscissa: Enkephalin concentration (M). Each value is the mean of 4-16 experiments. Vertical bars give S.E.M. **b** Concentration-response curve showing the effect of somatostatin on the electrically evoked contractions (●) of normal taenia coli and on the relaxations (○) following the addition of atropine (10^{-6} M) and guanethidine (5×10^{-6} M). The preparations were stimulated with 1 Hz for 3 s every 2 min. Ordinate: Percentage of the amplitude of the response obtained before addition of somatostatin. Abscissa: Somatostatin concentrations (M). Each value is the mean of 4-6 experiments. Vertical bars give S.E.M.

Discussion

Until recently, autonomic nerves were thought to comprise cholinergic and adrenergic elements. The peptide-containing nerves are now rapidly gaining acceptance as yet another subdivision of the autonomic nervous system (cf. Håkanson et al. 1980; Hökfelt et al. 1980; Sundler et al. 1980). To a large extent peptide-containing nerve fibres seem to originate from ganglia located within or in the vicinity of the target organ, suggesting that these nerves are engaged in local modulation of functional activity in the innervated organ, e.g. in local reflex arcs. Conceivably, the activity of these peptide-containing neurones is controlled by the central nervous system, via sympathetic or parasympathetic nerves. Unquestionably, peptide-containing nerve fibres form a prominent part of the intramural nerve supply of many organs in the body, but it remains to be established that the peptides act as neurotransmitters. Among the peptides identified in peripheral nerve fibres are SP, VIP, somatostatin and enkephalin. They all have powerful actions on a number of bodily functions (see Grossman 1974; Konturek 1976, 1978; Bury and Mashford 1977; Fahrenkrug 1979) and they are probably all stored in neurovesicles within the so-called p-type nerves. Hence, it is tempting to assume that they are released upon nerve stimulation to exert their actions locally. As recently discussed by Orrego (1979), the essential features of neurotransmitters are their presence inside neurovesicles, their release upon nerve stimulation and their interaction with specific receptors on adjacent cells. It follows that the actions of the putative transmitter must mimic faithfully those produced by the natural stimulus.

Peptide-containing nerve fibres are numerous in the taenia coli of the guinea pig, both in the smooth muscle and in the myenteric plexus, but from purely morphological observations it is not possible to decide whether a given peptide-containing neuronal system has sensory, associative or motor functions. Among the neuropeptides demonstrated in the taenia coli are those previously mentioned: SP, VIP, enkephalin, and somatostatin (see Furness and Costa 1980). The taenia coli-preparation was used to study the effects of these four neuropeptides on smooth muscle activity. VIP reduced the resting tension of the taenia and SP raised the resting tension. This is compatible with a role for VIP as an inhibitory and for SP as an excitatory neurotransmitter. This is also compatible with the view that both VIP neurones and SP neurones act as motor neurones (Fig. 13), particularly since both VIP nerve fibres and SP nerve fibres are fairly numerous in the smooth muscle. In addition, however, the response to electrical stimulation was affected by the presence of SP or VIP in the bath (Fig. 11). These changes in electrically evoked responses might be explained by the altered contractile state of the preparation induced by the peptides. Alternatively, this may reflect a neuromodulatory role for SP and VIP; some SP and VIP neurones in the myenteric plexus may represent interneurones. In Fig. 11 it can be seen that in the presence of SP the contractile response changed to a biphasic one: a relaxation followed by a contraction; and that in the presence of VIP the contractile response was enhanced. Both VIP fibres and SP fibres are numerous in the myenteric plexus where they are seen to surround cell bodies in a manner suggesting a role as interneurones. Such a role is strongly supported by previous observations (Hedquist and von Euler 1975; Katayama and

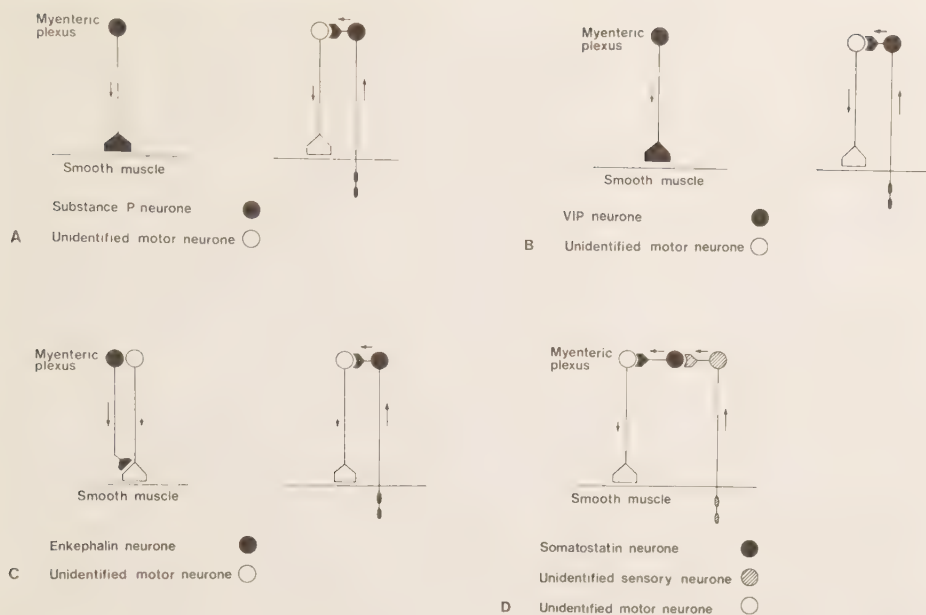


Fig. 13. Proposed roles for the various types of peptide-containing neurones in the control of motor activity in the taenia coli based on morphological as well as functional data. **A** SP neurones are probably motor neurones (excitatory). However, it cannot be excluded that SP neurones in addition act as sensory neurones and/or interneurons. **B** VIP neurones are probably motor neurones (inhibitory), although it cannot be excluded that some VIP neurones act as sensory neurones and/or interneurons. **C** Enkephalin neurones are interneurons or sensory neurones. In the former case they may act via axo-axonal mechanisms (which might explain why enkephalin fibres are numerous in the smooth muscle). **D** Somatostatin neurones probably act as interneurons in the myenteric plexus.

North 1978; Katayama et al. 1979; Holzer and Lembeck 1980; Jessen et al. 1980; Williams and North 1980) and it is not refuted by the present findings.

Somatostatin had no direct effects on the unstimulated taenia and only very weak effects on the electrically stimulated taenia. These observations together with the finding that the somatostatin-containing nerve fibres are found almost exclusively in the plexus (even here in moderate numbers) where they can be seen to ramify around nerve cell bodies support the view that the somatostatin neurones act as interneurons (Fig. 13).

The enkephalins, leu- and met-enkephalin, exerted a weak contractile effect on the unstimulated taenia and reduced the response to electrical stimulation. Enkephalin-containing nerve fibres were quite numerous in the smooth muscle. What then is the functional significance of these fibres, assuming that they act through the release of enkephalin? It is known from a number of studies that enkephalin effectively inhibits the release of several neurotransmitters, among them acetylcholine and noradrenaline (Enero 1977; Waterfield et al. 1977; Knoll et al. 1978; Uddman et al. 1980). Conceivably therefore, enkephalin serves to modify the release of transmitter from other nerve fibres in the smooth muscle, perhaps through axo-axonal contacts (cf. Clarke et al. 1979; Iversen et al. 1980). Within the smooth muscle, peptide-containing fibres, including those storing enkephalin, seem

to run close together in thin bundles, which may permit such contacts (see Schultzberg et al. 1980). Alternatively, the enkephalin nerve fibres in the smooth muscle are afferent elements involved in mediating sensory impulses to the myenteric plexus (Fig. 13). A functional significance of endogenous opiate receptor agonists in the peristaltic activity of the gut is supported by previous observations (Kromer and Pretzlaff 1979) and by the fact that the opiate receptor antagonist naloxone greatly affected the response of the taenia to electrical stimulation. The response to electrical stimulation was not altered by naloxone if atropine had been added to the bath, suggesting that naloxone, directly or indirectly, interferes with cholinergic transmission involved in the control of motor activity. If the enkephalins are implicated physiologically in the control of cholinergic transmission in the taenia, is unknown.

Recently, it was proposed that met-enkephalin and leu-enkephalin occur in separate but similarly distributed neuronal systems in the gut (Larsson et al. 1979). The physiological significance of this phenomenon is obscure, particularly since the two pentapeptides seem to have identical biological activity. However, although both met-enkephalin and leu-enkephalin have been identified in extracts of the brain (Hughes et al. 1975) and in peripheral tissues (Hughes et al. 1977), the identity of the material demonstrated by immunohistochemistry in each of the two nerve fiber populations has not been unequivocally established. On the whole, it seems likely that while at least one of the two neuronal systems demonstrated by immunohistochemistry contains met-enkephalin and or leu-enkephalin, the other one contains a chemically related compound with a biological activity different from that of the two "conventional" enkephalins.

In a recent study Cocks and Burnstock (1979) examined whether the inhibitory response of the taenia coli to electrical nerve stimulation following cholinergic and adrenergic blockade could be mediated by neuropeptides such as SP, VIP, somatostatin or enkephalin. SP was found to be excitatory (in agreement with numerous earlier observations on intestinal smooth muscle (for references, see Bury and Mashford 1977), somatostatin and enkephalin were inactive, while VIP induced a slow relaxation. For these reasons Cocks and Burnstock (1979) concluded that none of the neuropeptides were involved in the inhibitory response to electrical stimulation. However, the response to electrical stimulation includes both a relaxation and a contraction, and substance P may well be the excitatory transmitter. Burnstock et al. (1975), however, favoured the view that prostaglandins might be responsible since the rebound contraction was blocked by indomethacin, which is known to interfere with prostaglandin synthesis; we have not been able to confirm their observation with regard to the effect of indomethacin (Leander, unpublished observation). Further, Cocks and Burnstock (1979) suggested that VIP is an unlikely transmitter candidate for the inhibitory response because of its long latency of action. It is possible to argue, however, that this reflects physiologically irrelevant diffusion barriers in the preparation. A neuronal release of VIP giving high local concentrations may result in effects along a time scale quite different from the one seen after administration of exogenous VIP. Also, it may be argued that electric field stimulation probably releases a whole array of neurotransmitters, excitatory as well as inhibitory, each working along its own time scale. The mechanical response recorded is the net result of the actions of all these

transmitters and the time course of this response does not necessarily reflect the time course of action of a single neurotransmitter.

In fact, very recently Furness and Costa have argued in a series of publications (Costa et al. 1980a, b, c; Furness and Costa 1979, 1980) that various peptide-containing nerves of the intestine are involved in the control of peristalsis and that the neuropeptides act as transmitters. From morphological observations alone they suggested that SP would act on smooth muscle and on nerve cells of the myenteric plexus (Costa et al. 1980a). The VIP neurones of the myenteric plexus were found to project in an anal direction to supply primarily the circular muscle of the gut wall, an observation consistent with these neurones being "inhibitory enteric nerves" (Furness and Costa 1979). Also the somatostatin neurones of the myenteric plexus projected in an anal direction. The somatostatin fibres were seen to contact other neurones in the myenteric plexus, suggestive of a role as interneurones in a descending pathway (Costa et al. 1980c).

In summary: Peptide-containing nerve fibres, among them SP fibres, VIP fibres, enkephalin fibres and somatostatin fibres, represent a prominent proportion of the intramural nerve elements of the gut. There is mounting evidence that the four neuropeptides studied act as transmitters with direct or indirect effects on smooth muscle activity. All of them seem to be stored in neurovesicles, and while the effects of VIP and SP mimic smooth muscle responses induced by electrical field stimulation, somatostatin and enkephalin may act by modulating the activities of other neurones. On the whole, SP neurones and VIP neurones seem to act primarily by affecting the smooth muscle cells directly (motor neurones). Somatostatin neurones may act preferentially as interneurones and enkephalin neurones as interneurones or sensory neurones.

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Short title: Neuropeptides in the intestine

Neuronal Cholecystokinin, Gastrin-releasing Peptide, Neurotensin and β -Endorphin in Guinea-Pig Intestine. Distribution and Possible Motor Functions.

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SUMMARY

The guinea-pig intestine was found to harbour nerve fibers containing immuno-reactive cholecystokinin (CCK), gastrin-releasing peptide (GRP), neurotensin or β -endorphin. Such fibers occurred in the myenteric and submucosal plexuses and in the smooth muscle. GRP and CCK fibers in addition occurred in the mucosa. Following colchicine pretreatment nerve cell bodies in the myenteric plexus displayed CCK, GRP or β -endorphin immunoreactivity. CCK immunoreactive cell bodies were found also in the submucosal plexus. Neurotensin immunoreactive cell bodies could not be detected. The presence of immunoreactive nerve cell bodies in intramural plexuses indicates that CCK, GRP and β -endorphin containing fibers are intrinsic to the gut wall. GRP, neurotensin and β -endorphin were identified in extracts of smooth muscle by immunochemical and chromatographic analysis.

CCK-8, GRP and neurotensin caused a concentration-dependent contraction of the smooth muscle of the isolated taenia coli. Tetrodotoxin did not affect the responses to GRP and neurotensin, suggesting that these two peptides act directly on smooth muscle receptors. The effect of CCK-8 on the other hand is partly mediated by the release of acetylcholine, since atropine reduced the CCK-8 induced contractile response by about 20% and tetrodotoxin by 45%. The substance P (SP) antagonist, (D-Pro², D-Trp^{7,9})-SP, was ineffective. In addition, CCK-8 enhanced the SP-mediated contractile response to electrical stimulation but not that mediated by acetylcholine. GRP and neurotensin did not enhance the contractile responses. β -Endorphin had no effect on the tension of the muscle but reduced both cholinergic and SP-mediated neuronal responses through a naloxone-sensitive mechanism.

Key words: Neuropeptides, Autonomic nerves, Smooth muscle, Gut innervation, Cholecystokinin octapeptide, Gastrin-releasing peptide, Bombesin, Neurotensin, β -Endorphin.

INTRODUCTION

Neuropeptides in the gut wall, in particular substance P (SP), vasoactive intestinal polypeptide (VIP), enkephalin and somatostatin, have attracted much attention during recent years. Most of the work has been devoted to the distribution and structural organization of their polarities and projections (8, 18, 44, 47). Considerably less information is available on the functional roles of the neuropeptides, although there is growing evidence that they act as neurotransmitters and/or neuromodulators participating in the regulation of gut motility (for references see 15, 19, 21, 32, 33). The present report deals with four recently recognized neuropeptides in the gut wall, namely cholecystokinin octapeptide (CCK-8) (10, 11, 31, 40, 41), gastrin-releasing peptide (GRP) (12, 36, 53), neurotensin (44) and β -endorphin (51). These peptides were examined with respect to their distribution in the guinea-pig intestine and their effects on the motor activity of the isolated taenia coli. Our observations suggest that these four neuropeptides are involved in the regulation of the activity of gut smooth muscle.

MATERIALS AND METHODS

Immunocytochemistry

Thirty guinea pigs of both sexes weighing 250-350 g were used. Ten were anaesthetized with diethyl ether and perfused via the heart with an ice-cold solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2, for 5 min. Two of them were given colchicine (10 mg/kg) intraperitoneally and killed 24 h later. Specimens from duodenum, jejunum, ileum and colon were immersed in the fixative for 10-12 h and thoroughly rinsed in buffer containing 5-25% sucrose. The specimens were frozen on dry ice and sectioned in a cryostat at 10-20 μ m. In addition, small strips of the longitudinal smooth muscle with attached myenteric plexus were dissected out from twenty guinea pigs under a dissection microscope and stretched on microscope slides. These whole mounts were then fixed in a solution of picric acid and formaldehyde in 0.1 M phosphate buffer (pH 7.2), dehydrated, cleared in xylene and hydrated (7). Cryostat sections and whole mount preparations were processed for the immunocytochemical demonstration of cholecystokinin, GRP, neurotensin, or β -endorphin using previously characterized rabbit antisera (Table 1). The site of the antigen-antibody reaction was visualized by the indirect immunofluorescence method of Coons et al. (6) or the peroxidase-antiperoxidase (PAP) method of Sternberger (45). For controls we used antisera which had been inactivated by the addition of excess amounts of antigen (10-100 μ g/ml diluted antiserum). Cross reactivity with other peptides or proteins con-

Table I. Characteristics of the antisera used.

Antisera against	Code No.	Antigenic site	Immunofluorescence	Working dilution	Source	References
1. Gastrin/CCK	4562	COOH-terminus	1:640	1:5120	Prof. J.F. Rehfeld	35
2. GRP	6902	COOH-terminus	1:320	1:5120	Prof. N. Yanaihara	53
3. Neurotensin	HC-8	-	1:40	1:160	Dr. R.E. Carraway	46
4. β -Endorphin	7762	NH ₂ -terminus	1:40	1:80	own	2
	7763	COOH-terminus	1:40	1:80	own	2

Comments:

1. This antiserum recognizes the COOH-terminal pentapeptide common to gastrin and CCK and does not discriminate at the immunocytochemical level between gastrins and CCKs.
2. This antiserum is directed against the COOH-terminal portion of bombesin and when used for immunocytochemistry recognizes also the mammalian analogue gastrin-releasing peptide (GRP) (53).
3. HC-8 recognizes the COOH-terminal hexapeptide of neurotensin in a radioimmunoassay system (4, 5).
4. Antiserum 7763 cross-reacts with β -lipotropin, whereas antiserum 7762 does not.

taining amino acid sequences recognized by the different antisera cannot be excluded. Therefore, it would be appropriate to refer to the immunoreactive material as CCK-like, GRP-like, neurotensin-like or β -endorphin-like, respectively. For brevity, however, the immunoreactive nerve fibers are referred to as CCK-, GRP-, neurotensin- or β -endorphin-fibers.

Immunochemistry

For the determination of GRP and neurotensin mucosal and smooth muscle specimens from guinea-pig duodenum and colon were weighed and boiled in re-distilled water (0.1 g wet weight per ml) for 2 min. Following the addition of 3 M acetic acid to a final conc. of 0.5 M, the tissues were homogenized (Polytron, 60 sec). After centrifugation at 2000 x g for 15 min the supernatants were collected and lyophilized. Following resuspension in 0.5 M acetic acid the extracts were again centrifuged and the supernatants were passed through disposable C₁₈-Sep-Pak cartridges (Waters). Each Sep-Pak was washed with 10 ml 0.5 M acetic acid, which removes impurities such as inorganic salts and low molecular weight organic compounds. Peptides were eluted with 10 ml of a solution of 40% acetonitrile in water. The eluate was lyophilized, resuspended in 500 μ l of water and analysed by radioimmunoassay in serial dilutions or subjected to high performance liquid chromatography (HPLC). The HPLC system consisted of a Waters Model 204 liquid chromatograph equipped with a Model U6K injector, two Model 6000 A pumps, a Model 660 solvent programmer, a wavelength detector (210 nm) and a Model 440 absorbance detector (280 nm). A reverse phase column of μ -Bondapak C-18 (Waters) was used. The solvent system was composed of acetonitrile and 0.1 M phosphate buffer, pH 3.5, with an 18-35% linear gradient of acetonitrile over 60 min at a flow rate of 1.0 ml per min. Fractions of 0.5 ml were collected over a period of 60 min and assayed for GRP-like or neurotensin-like immunoreactivity. The neurotensin antiserum (HC-8) was a kind gift from Dr. R.E. Carraway, Harvard Medical School, Boston, Mass., USA. It has been characterized in detail elsewhere (4, 5). The bombesin/GRP antiserum was raised against 1,5-difluoro-2,4-dinitrobenzene-coupled bombesin-BSA conjugate (48). It cross-reacts with GRP to about 30% (37). Synthetic neurotensin and Tyr⁴-bombesin (Peninsula, San Carlos, USA) were iodinated with ¹²⁵I by a chloramine-I method (25). The neurotensin tracer was purified by reverse phase HPLC using μ -Bondapak C-18 as the stationary phase and acetonitrile: 0.1 M phosphate buffer, pH 3.5, (23:77 v/v) as the mobile phase (retention volume for neurotensin was 8 ml, for the tracer 16 ml). The eluate was scanned for radioactivity and absorbance (280 nm). The fractions containing ¹²⁵I-neurotensin were diluted 1:10 with 0.05 M phosphate buffer, pH 7.5, containing 0.25% human serum albumin, 0.05% sodium azide and 500 KIE Trasylol^(R)

(Bayer, Leverkusen, FRG) per ml and stored at -20°C .

The bombesin tracer was purified by reverse phase HPLC using μ -Bondapak C-18 as the stationary phase and acetonitrile: 0.1 M phosphate buffer, pH 3.5, (20:80 v/v) as the mobile phase. The eluate was scanned for radioactivity and absorbance (280 nm). The fractions containing ^{125}I -(Tyr⁴)-bombesin were diluted 1:10 with 0.05 M phosphate buffer, pH 7.5, containing 0.25% human serum albumin, 0.05% sodium azide and Trasylol^(R), 500 KIU/ml, and stored at -20°C . Bombesin-like peptides were determined by radioimmunoassay as described in detail elsewhere (37) and neurotensin-like peptides were determined as described by Carraway and Leeman (4, 5). Briefly, 200 μl antiserum (diluted 1:14000) was incubated with 100 μl standard or biological sample for 24 hours at $+4^{\circ}\text{C}$. 200 μl of ^{125}I -neurotensin (10,000 cpm) was added and the incubation continued for 24 hours at $+4^{\circ}\text{C}$. Antibody-bound tracer was separated from free tracer by adding 500 μl activated charcoal suspension. The tubes were incubated for 30 min at $+4^{\circ}\text{C}$ and centrifuged at $1700 \times g$ for 15 min at $+4^{\circ}\text{C}$. The supernatants were analyzed for radioactivity in a NEN 1600 gamma counter with a counting efficiency of 80%.

For the radioimmunoassay of β -endorphin the tissue specimens were weighed, minced and glass-homogenized in 5 ml cold 1 M acetic acid containing 20 mM HCl, 0.01% phenylmethylsulfonyl fluoride, 0.01% pepstatin A (Protein Research Foundation, Osaka, Japan) and Trasylol^(R), 500 KIU/ml. The homogenates were centrifuged at $5000 \times g$ at $+4^{\circ}\text{C}$ for 20 minutes and the supernatants were lyophilized. The lyophilized material was redissolved in 2-3 ml 0.05 M phosphate buffer, pH 7.5, containing 1% human serum albumin, 0.4% β -mercaptoethanol and Trasylol^(R), 500 KIU/ml. The samples were again centrifuged at $5000 \times g$ at $+4^{\circ}\text{C}$ for 10 minutes before analysis in serial dilutions using ^{125}I - β -endorphin as tracer and antiserum 7762 (final dilution 1:10000) (2). Alternatively, the extracts were subjected to gel chromatography (see below) before radioimmunoassay. Recovery of authentic peptide in the extraction was 70-80%. The results are presented uncorrected for the loss in extraction. Gel chromatography was performed on a 0.9×50 cm column of Sephadex G-50 (Pharmacia, Sweden) equilibrated with 0.05 M phosphate buffer, pH 7.5, containing 1% human serum albumin, 0.4% β -mercaptoethanol and Trasylol^(R), 500 KIU/ml. The same buffer was used for elution (2.1 ml/h at $+4^{\circ}\text{C}$). Fractions of 0.7 ml were collected. The column was calibrated using dextran blue, ^{125}I - β -endorphin and Na^{125}I .

Motor Activity Studies

Investigations were performed on isolated taenia coli preparations of male guinea-pigs, weighing 300-500 g. The animals were stunned by a blow on the

head and exsanguinated. Taenia strip preparations, consisting of longitudinal smooth muscle and attached myenteric plexus, were dissected from the caecum as described by Burnstock et al. (3) and placed in a modified Krebs' solution (for composition see below) at 4°C for about 1 h before start of motor activity studies. The taenia coli preparations were mounted vertically on a perspex holder in a 10 ml organ bath thermostated at 37°C. One end was attached to a rigid support and the free end connected to a lever via a spring to a Grass FT 03 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. In all experiments the load on the muscle was set at 0.2 g. Platinum ring electrodes were placed around the muscle with a constant distance between the rings of 10 mm. The electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (10-15 V over the electrodes; 0.5 - 1 msec duration). The muscle strips were stimulated with trains of pulses lasting 3-5 s and with a frequency of 1-5 Hz, one train every 2 min. The mechanical activity of the preparation was recorded continuously throughout the experiment on a Grass model 7 or Grass model 5 Polygraph. The bathing fluid was a modified Krebs solution with the following composition (in mM): NaCl 133; NaHCO₃ 16.3; KCl 4.7; MgCl₂ 1.0; NaH₂PO₄ 1.4; CaCl₂ 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 5% CO₂ in O₂ giving a pH of 7.2-7.3. Concentration-response curves were constructed by adding step-wise increasing amounts of CCK-8, bombesin, GRP, neurotensin or β -endorphin to the bath. The bath was rinsed between each addition. Care was taken not to test the next concentration of peptide until the effect of the preceding one had been washed away.

Chemicals

Sulfated CCK-8 (Sincalid^(R)) was a kind gift from Squibb, New Brunswick, N.J., USA. Synthetic bombesin, GRP, β -endorphin, neurotensin and SP were purchased from Peninsula, San Carlos, Ca., USA. ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP was generously provided by Dr. J. Hörig, Ferring, Kiel, FRG. Tetrodotoxin (TTX) was provided by Sankyo, Tokyo, Japan. Naloxone chloride was from Winthrop, USA, atropine sulphate from Vitrum, Stockholm, Sweden, carbachol from Merck, Darmstadt, FRG, guanethidine from CIBA-Geigy, Basle, Switzerland, and colchicine from BDH, Poole, England.

RESULTS

Occurrence and Distribution of Immunoreactive Neurons and Nerve Fibers

All four neuropeptides occurred in nerve fibers in the myenteric plexus and in the smooth muscle. Generally, they were more numerous in the ileum

Small intestine

Large intestine

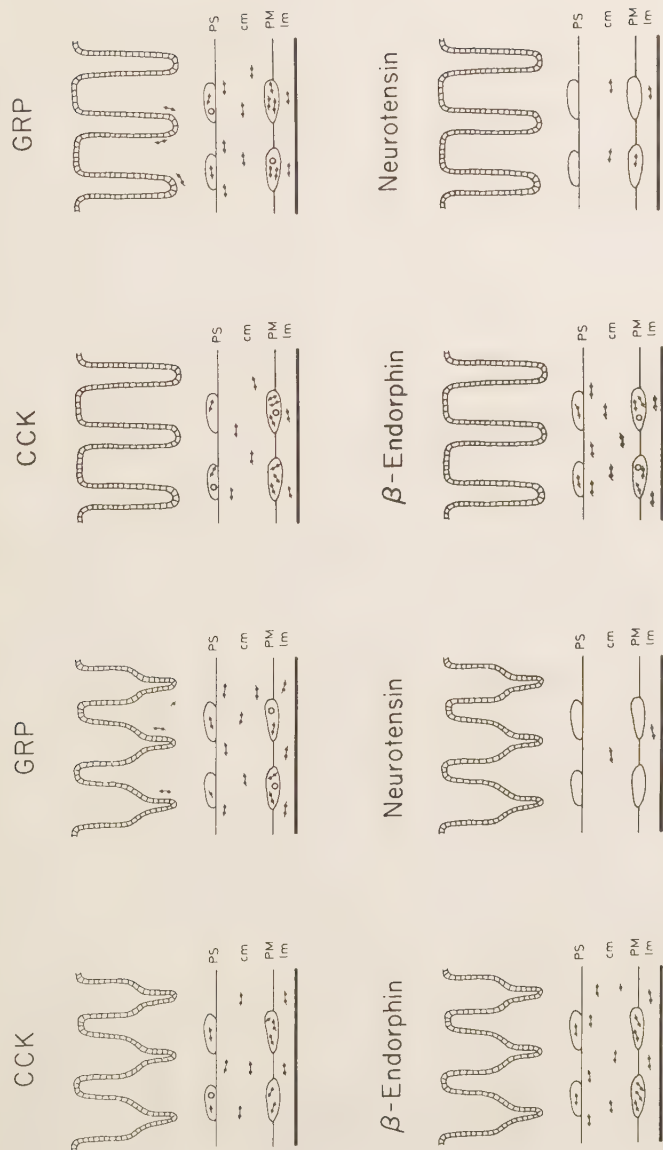


Fig. 1. Schematic outline of the topographical distribution and relative frequency of nerve fibers (—●—) and nerve cell bodies (o) displaying cholecystokinin (CCK), gastrin releasing peptide (GRP), β -endorphin or neurotensin immunoreactivity in the small and large intestine. No attempts were made to illustrate how the neurones project. PS = submucosal plexus, PM = myenteric plexus, cm = circular muscle, lm = longitudinal muscle.

and colon than in the duodenum and jejunum. However, the different types of nerve fibers differed somewhat with respect to their regional distribution (Table II). Also their topographic distribution differed as outlined in Table III and Fig. 1.

Table II. Regional distribution and relative frequency of different peptide-containing nerve fibers in the gut wall.

Peptide	Duodenum	Jejunum	Ileum	Colon
Gastrin/CCK	(+)	++	+++	+
Neurotensin	0	0	(+)	(+)
GRP	++	++	+++	++
β -Endorphin	++	++	++	++

0 absent, (+) rare, + few, ++ moderate number, +++ numerous.

Nerve fibers containing immunoreactive gastrin/CCK were moderate in number in the myenteric plexus ; they were found scattered in the smooth muscle (Fig. 2) and a few fibers occurred also in the submucosa and mucosa. Following



Fig. 2. Whole mounts of ileum, PAP method. (a) longitudinal muscle and (b) myenteric plexus. CCK-immunoreactive nerve fibers ramify around bundles of smooth muscle (a) (x 250) and form a dense network in the myenteric plexus. Immunoreactive nerve cell body at arrow. (b) (X 325).

Table III. Topographic distribution and relative frequency of peptidergic nerve fibres and cell bodies in their respective region of predominance.

Neuropeptide	Nerve Fibres		Nerve Cell Bodies	
	Smooth muscle	Myenteric Plexus	Myenteric plexus	Submucosal plexus
1. Gastrin/CCK	++	+++	+	+
2. Gastrin-releasing peptide	++	+++	+	+
3. Neurotensin	(+)	+	0	0
4. β -Endorphin	++	++	+	0

0, absent, + few, ++ moderate number, +++ numerous

colchicine treatment weakly immunoreactive nerve cell bodies could be seen in low number in the myenteric and submucosal plexuses.

GRP-immunoreactive fibers were fairly numerous in the myenteric plexus and moderate in number in the smooth muscle (Fig. 3). In the plexus the

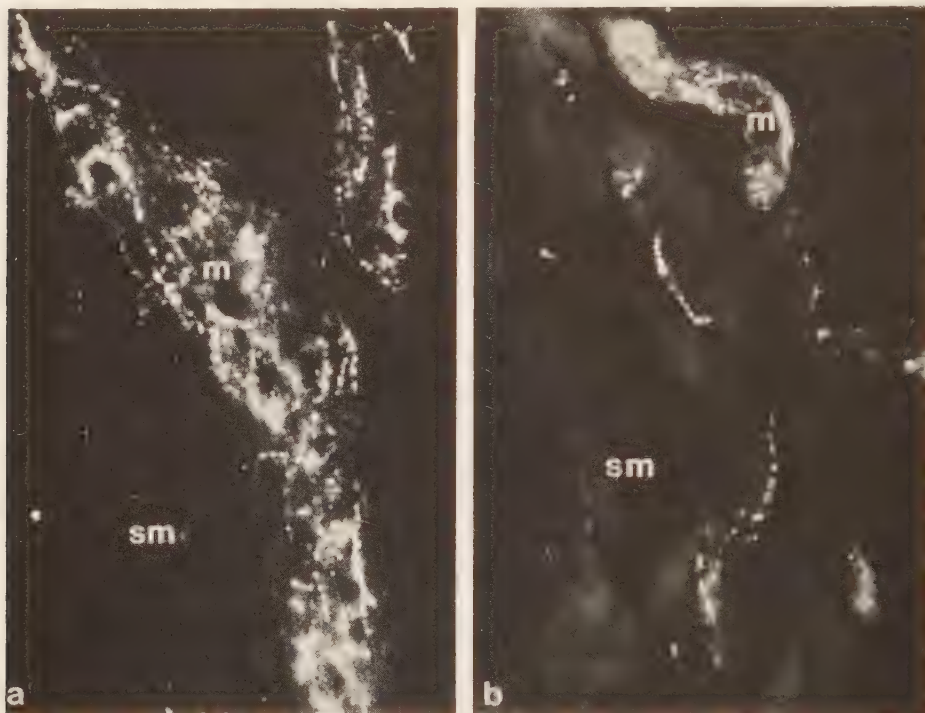


Fig. 3. Whole mount of jejunum (a) and cryostat section of taenia coli (b). Immunofluorescence. GRP fibers in the myenteric plexus (m) and in the smooth muscle (sm) (X 350).

fibers were sometimes seen to surround non-immunoreactive nerve cell bodies. Few, scattered fine-varicose fibers were observed in the mucosa and the submucosal plexus. Weakly immunoreactive nerve cell bodies occurred in low number in the myenteric plexus. After colchicine treatment the cell bodies displayed moderate to intense immunoreactivity.

Immunoreactive neurotensin was detected in a few scattered nerve fibers in the myenteric plexus and smooth muscle (Fig. 4). No immunoreactive nerve cell bodies were observed.

Immunoreactive β -endorphin occurred in rather many nerve fibers in the myenteric plexus and smooth muscle of the gut wall (Fig. 5). Following colchicine treatment immunoreactive nerve cell bodies could be observed in the myenteric plexus. The two β -endorphin antisera revealed the same histochemical picture.

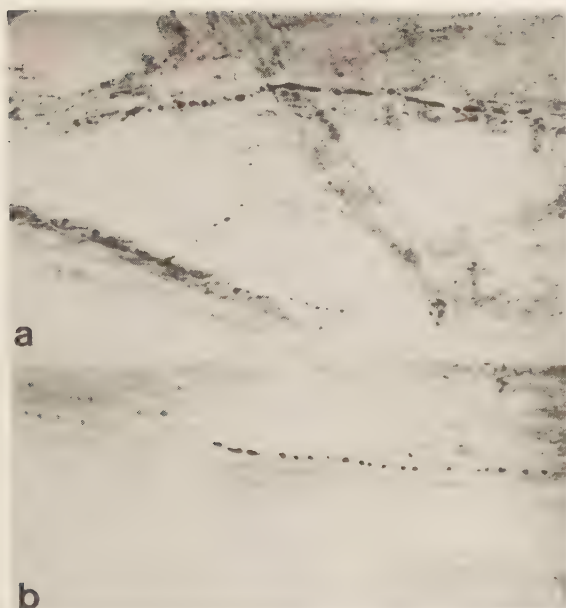


Fig. 4. Whole mounts of ileum. PAP-method. Scattered varicose neurotensin fibers in the longitudinal muscle (a) and circular muscle (b) (X 450).



Fig. 5. Whole mounts of ileum. β -Endorphin fibers in the smooth muscle (X 350).

Chemical Determination and Analysis of the Neuropeptides

The concentrations of the peptides in guinea-pig intestine are given in Table IV. The smooth muscle layers both in the small and large intestine

Table IV. Concentration of different neuropeptides in the duodenum and colon of the guinea pig

		β -Endorphin ng eqv/g	GRP ng eqv/g	Neurotensin pg eqv/g
Duodenum	Mucosa	6.1 $\pm$ 0.7	14 $\pm$ 3	665 $\pm$ 114
	Smooth muscle	33.0 $\pm$ 5.5	618 $\pm$ 60	376 $\pm$ 134
Colon	Mucosa	5.1 $\pm$ 0.5	29 $\pm$ 3	355 $\pm$ 19
	Smooth muscle	12.7 $\pm$ 1.7	150 $\pm$ 19	78 $\pm$ 10

Mean $\pm$ S.E.M. (n = 4)

were notably rich in GRP. The mucosa contained only little. Also β -endorphin was more abundant in the smooth muscle than in the mucosa. Neurotensin, on the other hand, predominated in the mucosa; even in this location the concentrations were comparatively low. HPLC of extracts of intestinal smooth muscle revealed one neurotensin-immunoreactive and one GRP-like compound that co-eluted with authentic neurotensin and GRP, respectively (Fig. 6). Gel chromatography of extracts of guinea-pig ileum revealed one major β -endorphin-immunoreactive peak with the same elution position as synthetic β -endorphin (Fig. 7).

Motor Effects of the Neuropeptides on the Isolated Taenia Coli

Effects on Resting Tension: CCK-8 caused a concentration-dependent contraction of the smooth muscle (Figs 8, 9). Atropine (10^{-6} M) reduced the maximum contractile response by 20% whereas (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP was ineffective. The TTX-resistant response was approx. 55% of that evoked by CCK-8 in a control preparation (Fig. 8). GRP, bombesin and neurotensin produced concentration-dependent contractions that could not be blocked by either atropine, (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP or TTX (Figs 10,11,12). β -Endorphin had no effect on the resting tension (Fig. 13).

Effects on Neurally Evoked Motor Activity: Electrical stimulation at low frequency (1 Hz, 3s) evoked a contraction that could be blocked by atropine

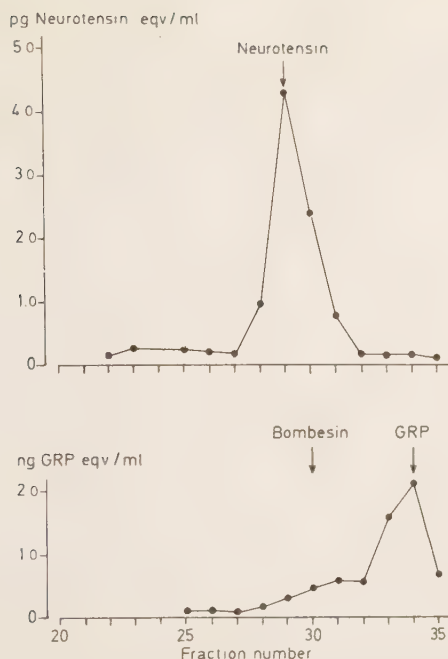


Fig. 6. HPLC elution profiles of GRP- and neurotensin-immunoreactive material in extracts of smooth muscle from guinea pig colon. The column was loaded with extract corresponding to 600 mg tissue wet weight. The elution positions of synthetic bombesin, GRP and neurotensin are indicated.

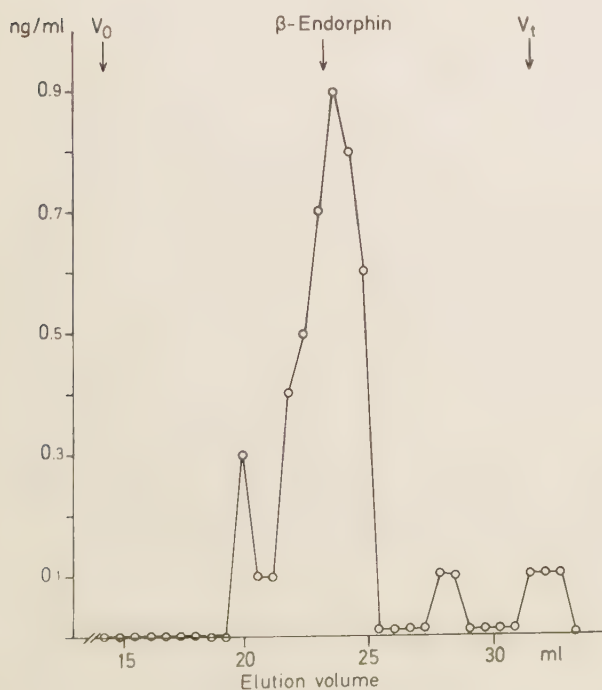


Fig. 7. Gel chromatographic elution profile of β -endorphin-immunoreactive components in an extract of small intestinal smooth muscle analyzed by gel filtration on Sephadex G-100 (for details see methods). The column was loaded with extract corresponding to 1 g tissue wet weight. The elution position of β -endorphin is indicated. V_0 = the elution volume of blue dextran, V_t = the elution volume of ^{125}I . Concentrations are given as ng equivalents of β -endorphin per ml.

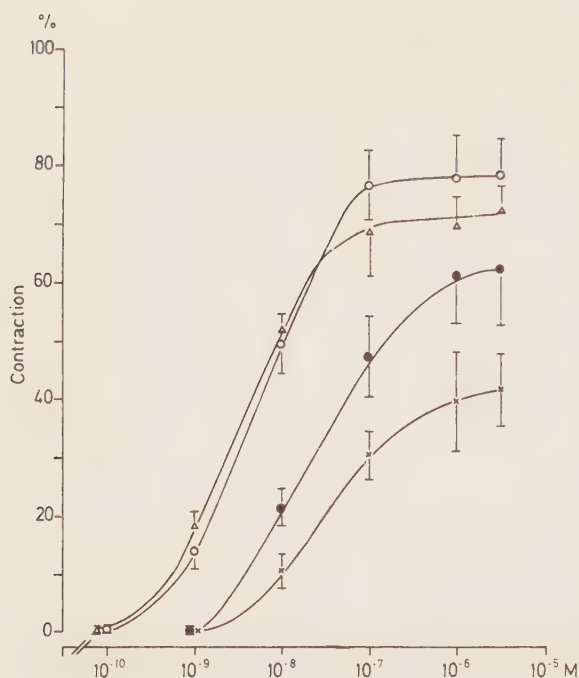


Fig. 8. Concentration-response curves showing the effects of CCK-8 alone (O) and in the presence of (D-Pro², D-Trp^{7,9})-SP (10⁻⁵ M) (Δ), atropine (10⁻⁶ M) (●), or TTX (10⁻⁶ M) (×) on the tension of the taenia coli. Ordinate: Increase in tension as a percentage of the contraction induced by carbachol (10⁻⁵ M). Abscissa: Molar concentration of CCK-8. Each value is the mean of 4-27 experiments. Vertical bars give S.E.M.

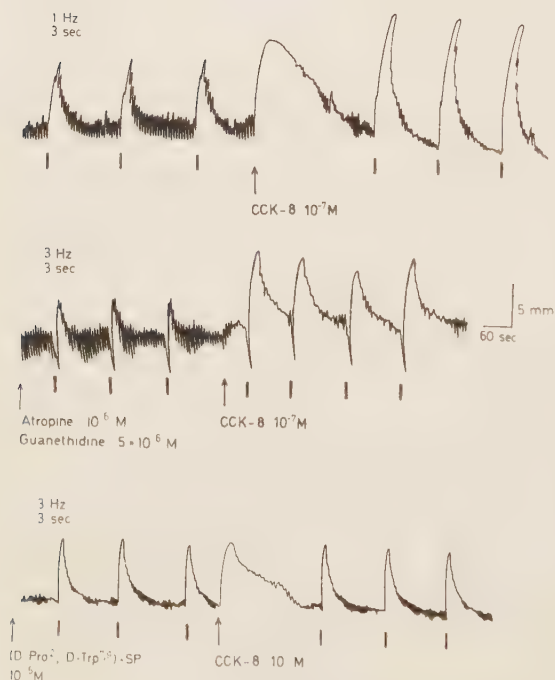


Fig. 9. Registrations showing the effect of CCK-8 on the tension of the taenia coli and on the amplitude of electrically evoked responses. The top panel shows the activity of a control preparation, the middle panel the activity of an atropine- and guanethidine-treated preparation while the one below shows the activity in the presence of (D-Pro², D-Trp^{7,9})-SP. Electrical stimulation is indicated by black rectangles. Note the increase in amplitude of electrically evoked responses in the control preparation and in the atropinized preparation following addition of CCK-8 and the absence of this potentiation in the presence of the SP antagonist.

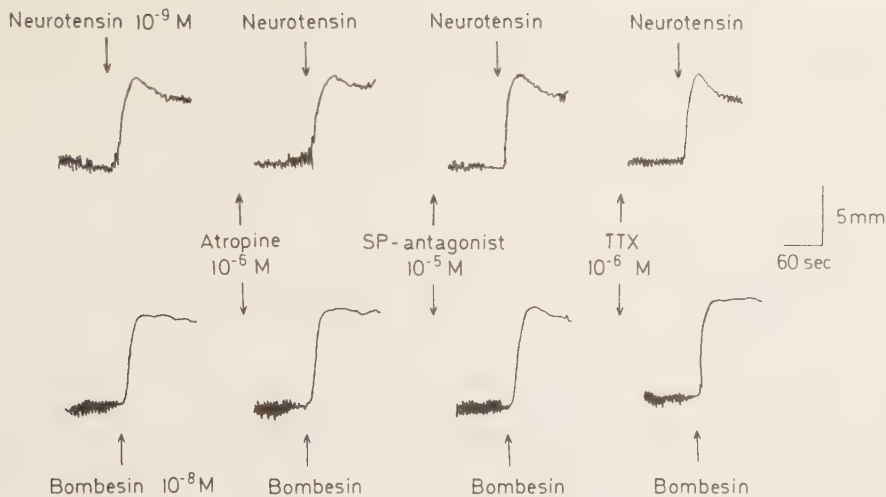


Fig. 10. Registrations showing the effect of neurotensin (upper panel) and bombesin (lower panel) on the tension of the taenia coli. The preparations were rinsed after each addition of peptide. The first administrations of peptide were made on control preparations, the second in the presence of atropine, the third administrations in the presence of atropine and the SP antagonist, (D-Pro², D-Trp^{7,9})-SP, and the fourth administration were made following the addition of TTX. Note that neither of the drugs tested affected the responses to neurotensin and bombesin.

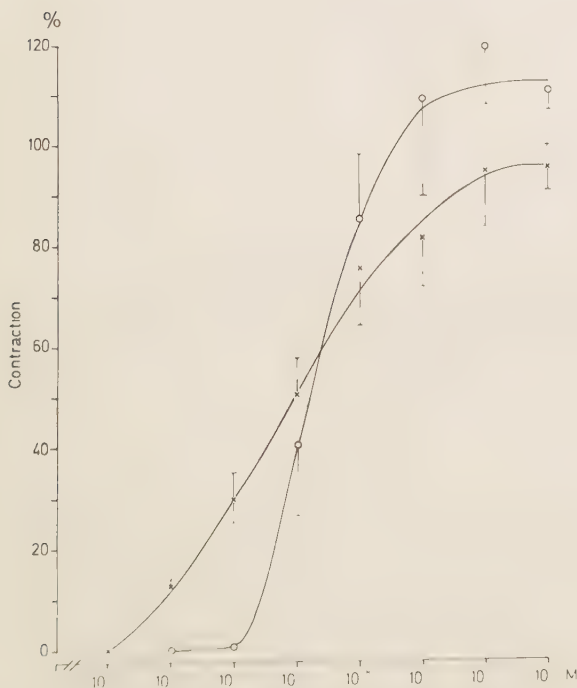


Fig. 11. Concentration-response curves showing the effects of neurotensin (X) and GRP (O) on the tension of the taenia coli. Ordinate: Increase in tension as a percentage of the contraction induced by carbachol (10^{-5} M). Ab-scissa: Molar concentrations of neurotensin and GRP. Each value is the mean of 4-10 experiments. Vertical bars give S.E.M.

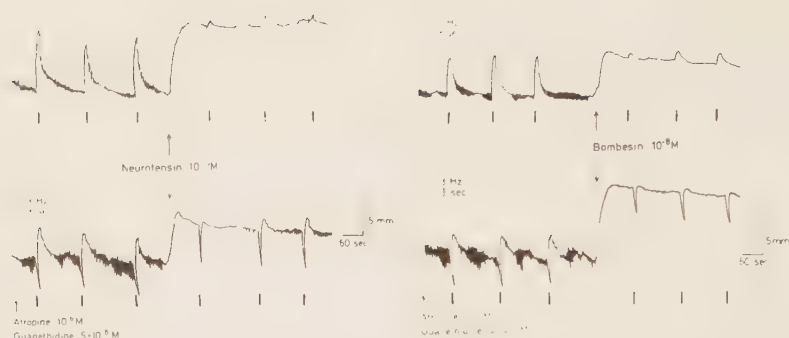


Fig. 12. Registrations showing the effect of neurotensin (left) and bombesin (right) on tension and electrically evoked responses in the taenia coli. The top panel of each pair shows the activity of a control preparation while the one below shows the activity in the presence of atropine and guanethidine. Electrical stimulation is indicated by black rectangles. Note the increased tension after addition of neurotensin (left) and bombesin (right) and the decrease in the electrically evoked contractile responses.

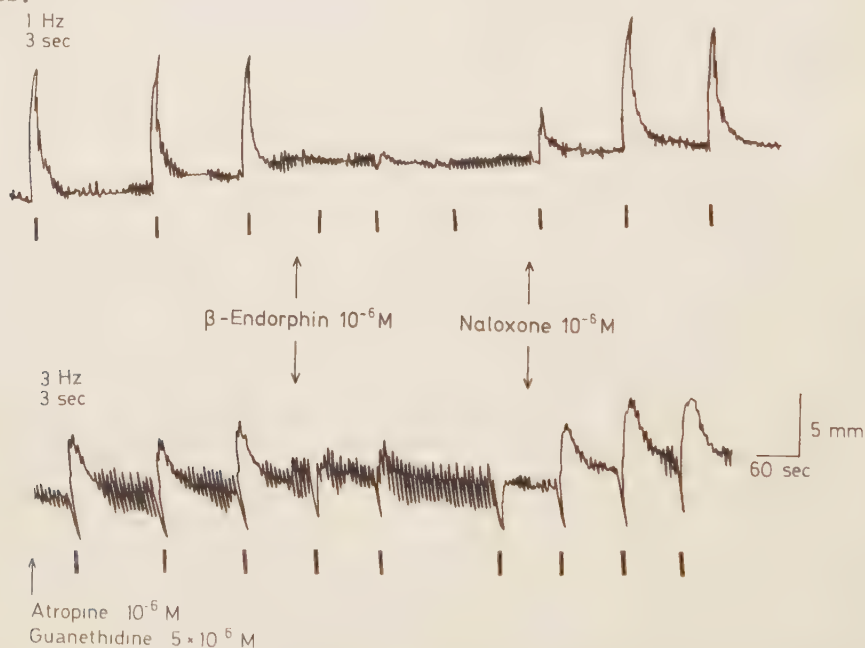


Fig. 13. Registrations showing the effect of β -endorphin on the amplitude of electrically evoked responses in the taenia coli. The top panel shows the activity of a control preparation while the one below shows the activity in the presence of atropine and guanethidine. Electrical stimulation is indicated by black rectangles. Note the decrease in electrically evoked contractions following addition of β -endorphin in both preparations and the ability of naloxone to block the effect of β -endorphin.

but not by the SP antagonist ($\underline{\text{D-Pro}}^2$, $\underline{\text{D-Trp}}^{7,9}$)-SP. Electrical stimulation at higher frequencies (3-5 Hz, 3-5 s) evoked a stronger contraction. In the presence of atropine and guanethidine the response was changed to an initial relaxation followed by a contraction upon cessation of stimulation. This contraction could be blocked by the SP antagonist (32). All electrically evoked responses could be blocked by TTX.

With or without atropine in the bath CCK-8 enhanced the contractile responses to both low and high frequency stimulation; the relaxation was not affected (Fig. 9). In the presence of ($\underline{\text{D-Pro}}^2$, $\underline{\text{D-Trp}}^{7,9}$)-SP CCK-8 did not enhance electrically evoked contractile responses.

GRP, bombesin and neurotensin reduced the electrically induced contractile responses but only in concentrations that raised the resting tension. The relaxation was not affected (Fig. 12).

β -Endorphin greatly reduced the atropine-sensitive contraction evoked by low frequency stimulation as well as the ($\underline{\text{D-Pro}}^2$, $\underline{\text{D-Trp}}^{7,9}$)-SP-sensitive contraction evoked by high frequency stimulation. The relaxation was not affected. The reduction of the contractions was blocked by naloxone (Fig. 13).

DISCUSSION

The present study demonstrates nerve fibers containing immunoreactive gastrin/CCK, GRP, neurotensin or β -endorphin in the gut of the guinea-pig. Neuronal CCK, GRP and neurotensin have previously been demonstrated immunocytochemically in the gut of rats and guinea-pigs (12, 31, 39, 44, 53), findings which have been verified immunochemically with respect to neurotensin (4, 5), CCK (26, 40) and GRP (36, 53). In the present study the results of radio-immunoassay were supplemented with chromatographic analysis of the immunoreactive material. The findings support the view that authentic GRP, β -endorphin and neurotensin exist in nerve fibers of the gut wall. Previous studies have indicated that the neuronal gastrin/CCK immunoreactive material represents predominantly CCK-8 (10, 11, 31, 40, 41). Fibers containing immunoreactive gastrin/CCK, GRP and β -endorphin occurred in a fair number whereas neurotensin immunoreactive fibers were scarce, in agreement with the immunochemical findings. The high levels of neurotensin in the mucosa reflect its presence in endocrine cells (46). The demonstration of immunoreactive nerve cell bodies in the myenteric plexus indicates that CCK-8, β -endorphin and GRP containing neurons are intrinsic to the gut wall. Neurotensin immunoreactive nerve cell bodies could not be detected, hence no conclusion can be drawn with respect to the origin of the neurotensin fibers.

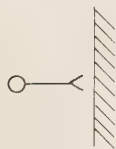
The gut wall is richly supplied with peptide-containing nerve fibers.

Several reports have described fibers containing VIP, SP, somatostatin, enkephalins and a peptide recognized by antibodies to avian pancreatic polypeptide (for references see 21, 22, 27, 34, 43). Obviously, the innervation of the gut is extremely complex, comprising a large number of neuronal systems. Although adrenergic and cholinergic nerves are generally thought to control the many diverse functions of the digestive tract, non-cholinergic, non-adrenergic nerve-mediated contraction and relaxation of gut smooth muscle have been demonstrated (1, 16). Recently, neuropeptides have emerged as attractive transmitter candidates for the mediation of such non-cholinergic, non-adrenergic neuronal effects. At present, however, the evidence for this view is largely circumstantial and based primarily on the existence of the neuropeptides in neurovesicles, their release upon nerve stimulation, and their characteristic bioactivity profiles. Many neuropeptides have pronounced effects on gut smooth muscle, and the isolated taenia coli is a convenient experimental model for evaluating their physiological roles. From the results of such experiments, neuropeptides may be classified in those that act directly on smooth muscle receptors (1) and those that act on neuronal receptors (2) (Fig. 14).

1) SP, GRP, CCK-8, neurotensin and VIP produce effects on the isolated taenia that are unaffected by the neuronal blocking agent TTX. They therefore belong to the group acting directly on smooth muscle receptors. SP, GRP, CCK-8 and neurotensin contract, while VIP relaxes the isolated guinea-pig taenia coli. Interestingly, neurotensin has been found to relax the rat ileum and guinea pig colon and to contract the guinea pig ileum and taenia coli (28, 30). The neurotensin-induced contraction of the guinea pig ileum resulted from a nerve-mediated, partly cholinergic process (29) whereas neurotensin was found to act directly on the taenia (28). GRP is a bombesin-like peptide recently isolated from mammalian non-antral stomach (36); bombesin-like peptides are known to contract smooth muscle in a number of tissues (13, 14). Bombesin and probably GRP as well are potent releasers of other neurohormonal peptides (see 13, 20), and Rökæus and McDonald (42) observed release of neurotensin-like material from the intestine following administration of bombesin. Such a mechanism does not seem to apply in the isolated taenia coli, since the contractile responses to both GRP and bombesin were TTX-resistant. The contractions produced by CCK-8 can be partly blocked by TTX, suggesting that to a minor extent CCK-8 acts directly on smooth muscle receptors.

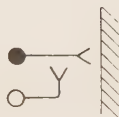
A specific, competitive SP receptor antagonist, (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP, antagonizes the neuronally mediated non-cholinergic, non-adrenergic contraction of the isolated guinea-pig taenia coli (32), thus supporting the

CHOLECYSTOKININ-8
 GASTRIN RELEASING PEPTIDE
 NEUROTENSIN
 SUBSTANCE P
 VASOACTIVE INTESTINAL PEPTIDE



NEUROPEPTIDES ACTING ON
 SMOOTH MUSCLE RECEPTORS:

CHOLECYSTOKININ-8
 β -ENDORPHIN
 ENKEPHALIN
 SOMATOSTATIN
 SUBSTANCE P



NEUROPEPTIDES ACTING ON
 NEURONAL RECEPTORS:

Fig. 14. Neuropeptides in the gut wall can be classified in 1) those that act on smooth muscle receptors and (2) those that act on neuronal receptors. The classification of the gut neuropeptides may differ with the species and the organ under study. SP, VIP, neurotensin, CCK-8 and GRP seem to belong to the first group. Their motor effects cannot be (completely) inhibited by tetrodotoxin, a drug known to block nervous conduction. Hence, these neuropeptides probably act directly on the smooth muscle cells. A number of other gut neuropeptides (CCK-8, SP, somatostatin, β -endorphin, and the enkephalins) seem to act indirectly, presumably via neuromodulatory effects exerted within the myenteric plexus or through axo-axonal contracts within the smooth muscle layer. Interestingly, SP and CCK-8 seem to belong to both categories.

view that SP is a motor excitatory transmitter in the gut. Specific antagonists to GRP, neurotensin and VIP are not available. Hence, their neurotransmitter role remains conjectural, although VIP has been proposed as a motor inhibitory transmitter in the gut (17, 19). From available results it seems that all contractile responses of the taenia coli to electrical field stimulation can be blocked by atropine and the SP receptor antagonist in conjunction, suggesting that no other motor excitatory neurotransmitter besides acetylcholine and SP are involved. It must be realized, however, that the motor response of the muscle to electric stimulation probably reflects the collective action of a number of excitatory and inhibitory transmitters, and that for instance, a relaxatory response to nerve stimulation might mask effects of excitatory transmitters released simultaneously with the inhibitory transmitters.

2) SP, enkephalins, CCK-8, β -endorphin and possibly somatostatin act on neuronal receptors to modulate neuronal activity. Of these CCK-8 and SP (both of which produce additional effects directly on smooth muscle receptors) act by increasing the contractile motor response to electric field stimulation, i.e. they are excitatory neuromodulators, whereas enkephalin, β -endorphin and somatostatin are inhibitory neuromodulators. In the isolated guinea-pig ileum SP potentiates the contractile response to electrical nerve stimulation (24), probably the cholinergic component since the response could be blocked by atropine. Gastrins and cholecystokinins are known to evoke release of acetylcholine from the myenteric plexus of guinea-pig ileum (9, 23, 49, 50). Hutchison and Dockray (26) have suggested that sulphated CCK-8 releases not only acetylcholine but also SP. This is unlikely in the case of the taenia since the SP antagonist did not affect the response of the taenia to CCK-8. The transmitter responsible for the atropine-resistant contraction produced by CCK-8 therefore remains to be identified. However, CCK-8 seems to potentiate the contractile response of the taenia to stimulation of SP-ergic nerve fibers. Neither SP nor CCK-8 affected the inhibitory neuronal systems of the gut wall. Enkephalin, and even more so β -endorphin, inhibited the contractile response to stimulation of cholinergic nerves and SP nerves; these effects were reversed by naloxone. β -Endorphin, like many other opiate agonists, effectively prevents the contractions produced by electrical stimulation in a longitudinal muscle strip or intact segment of guinea-pig ileum (52). Also the contractile response to neurotensin in these preparations is abolished (38). Neither enkephalin nor β -endorphin affected the inhibitory neuronal systems of the isolated taenia. Somatostatin, finally, has only marginal effects on neuronal responses in the guinea-pig taenia coli (33). The fact that different types of peptidergic nerve fibers

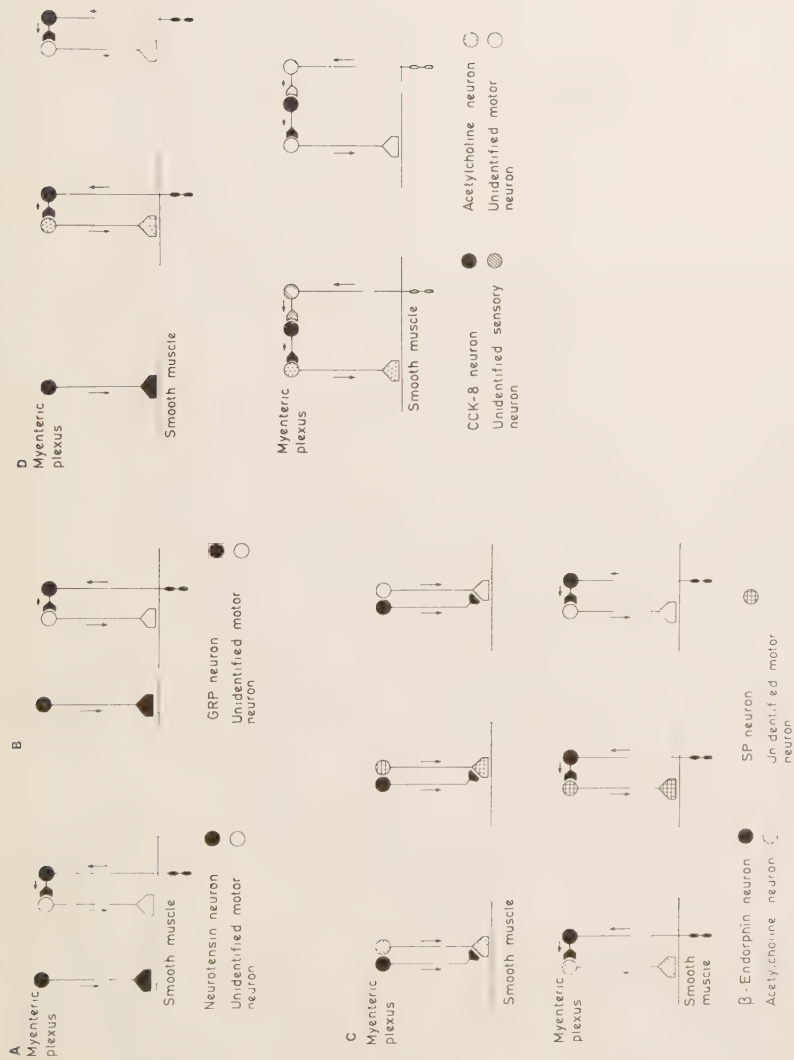


Fig. 15. Possible roles for the various types of peptide-containing neurons in the control of motor activity in the taenia coli based on morphological as well as functional observations. A and B. Neurotensin (A) and GRP (B) neurons are probably motor neurons (excitatory). However, it cannot be excluded that neurotensin and GRP neurons in addition act as sensory neurons and/or interneurons. C. β -Endorphin neurons are probably interneurons or sensory neurons. In the former case they may act via axo-axonal mechanisms, thus explaining the presence of β -endorphin fibers in the smooth muscle. D. Cholecystokinin-octapeptide (CCK-8) neurons may act both as motor neurons and as sensory neurons/interneurons. The CCK-8 induced contraction of the taenia coli is partly atropine-sensitive indicating the involvement of acetylcholine and an unidentified excitatory transmitter. The SP antagonist is without effect on the CCK-8 induced contraction but it may be noted that the SP antagonist counteracts the CCK-8 induced enhancement of the electrically evoked contraction.

often occur together in small bundles running along the smooth muscle fibers (44) may provide a morphological basis for interactions between them. The neuromodulatory effects demonstrated by many of the neuropeptides may well be exerted in such nerve bundles (via axo-axonal contacts) rather than in the intramural plexuses. Fig. 15 summarizes our conclusions on the conceivable functional roles of CCK-8, GRP, neurotensin and β -endorphins with respect to the motor activity of the guinea pig taenia coli.

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Neuronal substance P in the esophagus. Distribution and effects on motor activity

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Substance P-immunoreactive nerve fibres were fairly numerous in the lower esophagus of the guinea-pig and cat but few in the pig. They were particularly numerous in the myenteric and submucosal plexuses but could be detected also in the circular and longitudinal smooth muscle and in the muscularis mucosae. Only in the cat were SP-immunoreactive cell bodies detected, albeit in low number, in the myenteric plexus. Radioimmunoassay showed that the lower part of the cat esophagus contained approximately 10 times more immunoreactive SP than the upper part and that the muscle layer contained more SP than the mucosa. Motor effects of synthetic SP were studied on segments from circular smooth muscle of cat esophagus. SP contracted the smooth muscle and enhanced the response to electrical stimulation. These effects of SP could be blocked by the specific SP antagonist (D-Pro,² D-Trp^{7,9})-SP. The contractile response to electrical stimulation could be blocked by the cholinergic muscarinic blocker atropine and the opiate receptor agonist leu-enkephalin but not by the SP antagonist or by adrenergic blockers. Hence, the results suggest that cholinergic neurons innervate the circular smooth muscle, and that opiate receptor agonists suppress transmission in these neurons. Neuronal SP in the esophagus may serve to enhance the contractile responses of esophageal smooth muscle.

Key words: Substance P, esophagus, neuropeptides, autonomic innervation

Substance P (SP)-containing nerve fibres have a widespread distribution in the body (cf. Hökfelt et al. 1975, 1977, 1980; Sundler et al. 1980). Such fibres are numerous in the intestinal smooth muscle and in the myenteric and submucosal plexuses (Furness & Costa 1980, Hökfelt et al. 1980, Schultzberg et al. 1978, 1980, Sundler et al. 1980) and there is much experimental evidence that the majority of these fibres are intrinsic to the gut wall (Schultzberg et al. 1978, 1980, Franco et al. 1979, Costa et al. 1980, Jessen et al. 1980, Malmfors et al. 1980, 1981). Besides its anticipated role as a transmitter in primary sensory neurones (cf. Lembeck 1953, Otsuka & Konishi 1976, Lembeck & Holzer 1979,

Henry et al. 1980, Brodin et al. 1981a) SP may play a physiological role in the regulation of vascular and non-vascular smooth muscle activity (Pernow 1953, Hallberg & Pernow 1975, Hedqvist & von Euler 1975, Katayama et al. 1979, Yau 1978, Leander et al. 1981).

Previously, SP has been reported to increase the tone of the lower esophageal sphincter of the opossum (Mukhopadhyay 1978) and radioimmunoassay has revealed immunoreactive SP in the esophagus of several mammals (Nilsson & Brodin 1977, Holzer et al. 1980, Brodin & Nilsson 1981). We have examined the cellular localization and topographical distribution of immunoreactive SP in

the esophageal wall of guinea-pig, cat and pig and studied the effects of synthetic SP on the isolated circular smooth muscle of the cat esophagus.

MATERIAL AND METHODS

Immunocytochemistry

Tissue specimens were collected from the esophagus of adult guinea-pigs, cats and pigs, at least 5 of each species. Guinea-pigs were anesthetized with diethyl ether and cats with pentobarbitone (Mebumal®, ACO, Stockholm, Sweden) 40 mg/kg intraperitoneally. The guinea-pigs and cats were perfused via the heart with an ice-cold solution of 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Specimens were collected from various parts of the esophagus and immersed in the fixative for 24 h at 4°C. They were rinsed in a Tyrode solution containing 5% sucrose at 4°C for 24–48 h, frozen on dry ice and sectioned in a cryostat at –20°C. Material from adult pigs, obtained from a local slaughter-house, was frozen to the temperature of liquid nitrogen in a propane-propylene mixture and freeze-dried. The specimens were fixed by exposure to diethylpyrocarbonate vapor at 55°C for 3 h and embedded in paraffin *in vacuo*. Sections from paraffin-embedded specimens (6 µm in thickness) and cryostat sections (20 µm) were processed for the immunocytochemical demonstration of SP using antisera raised in rabbits against synthetic bovine SP conjugated to serum albumin. The antisera (code No. K-25 and SP-8) have previously been used in immunocytochemical studies (Nilsson et al. 1975a, Håkanson et al. 1977, Sundler et al. 1977, Alumets et al. 1980). Antiserum K-25 is directed against the C-terminal end of SP and shows negligible cross-reaction with other naturally occurring peptides with the exception of physalaemin at the antiserum dilutions used in radioimmunoassay (Nilsson et al. 1977, Brodin et al. 1981b). When used in immunocytochemistry this antiserum cross-reacts with both bombesin and physalaemin (Brodin et al. 1982). Antiserum SP-8 (kind gift from Dr P. Emson, MRC, Cambridge, England) is also directed against the C-terminal end and cross-reacts with physalaemin but not with bombesin at the immunocytochemical level (cf. Brodin et al. 1981b). For immunofluorescence antiserum K-25 was diluted 1:40 and SP-8 1:80 and for the peroxidase anti-peroxidase (PAP) procedure (Sternberger 1979) antiserum SP-8 was diluted 1:320. In the immunofluorescence procedure the site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate-labelled goat anti-rabbit IgG (Coons et al. 1955) in dilution 1:20. In the PAP procedure unlabelled goat anti-rabbit IgG (diluted 1:30) was applied as a second layer, followed by incubation with PAP complex (Dako, Copenhagen, Denmark), in dilution 1:320. Sections incubated with antiserum inactivated by the addition of excess antigen (100 µg of synthetic SP per ml diluted antiserum) were used as controls. Immunofluorescence was examined in a fluorescence microscope equipped with an epi-illumination system and filters selected to give peak excitation at 490 nm. PAP-stained sections were examined in a light microscope. For brevity nerve fibres displaying SP immunoreactivity are referred to as SP fibres.

Radioimmunochemical determination of SP

Tissue specimens (0.5–1.0 g) from the upper, middle and lower parts of the esophagus were dissected out from 5 cats and divided into mucosa and muscle. The specimens were frozen on dry ice and stored at –20°C until extracted. They were then weighed, minced in partly frozen condition and extracted in 10 volumes of 1.0 M acetic acid in a hot water bath for 10 min. Following centrifugation the supernatants were lyophilized and then redissolved in assay buffer immediately prior to radioimmunoassay (Nilsson et al. 1975b, 1977, Brodin et al. 1981b). The SP antiserum (K-25) was used in a final dilution of 1:350 000, and ¹²⁵I-[Tyr⁴]-SP served as tracer. The extracts were assayed in duplicates at two or three dilutions. The smallest amount detectable was 2.5×10^{-12} g (1.85×10^{-15} mol). The antiserum cross-reacts less than 5% with physalaemin (Nilsson et al. 1977).

Motor activity studies

The experiments were performed on isolated esophageal circular smooth muscle from 23 cats. The esophagus was cut open from the esophago-gastric junction, the mucosa was removed with a pair of scissors and the muscle was divided into 8 consecutive strips (4 × 15 mm) perpendicular to the longitudinal axis of the preparation. The muscle strips were placed over night in cooled Ringer solution of the following composition in mM: NaCl 133; NaHCO₃ 16.3; KCl 4.7; MgCl₂ 1.0; NaH₂PO₄ 1.4; CaCl₂ 2.5; glucose 7.8. Each preparation was mounted vertically on a muscle holder in a 7 ml organ bath thermostated at 37°C and bubbled with a gas mixture of 7% CO₂ in O₂ giving a pH of 7.2–7.3. The free end was attached to a lever connected via a spring to a Grass FTO3 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. The load on the muscle was 0.5 g. Platinum ring electrodes were placed around the muscle with a constant electrode distance of 5 mm. The electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (15–20 V over the electrodes, 0.5–1.0 ms). The mechanical activity of the preparation was recorded continuously on a Grass model 7 or model 5 Polygraph. The preparations were allowed to equilibrate in the bath for 60 min before experimentation started. At the start of each experiment the viability of the preparation was tested by stimulating with trains of pulses (1 Hz for 3 s) at 2 min intervals. The SP concentration-response curve was constructed by adding step-wise increasing amounts of SP to the bath. Between each addition the bath was rinsed and the next dose was not added until the effect of the preceding one had disappeared. Below 10^{–7} M three concentrations of SP were tested on each muscle; above 10^{–7} M only one concentration was tested per muscle. The increase in tension induced by SP was expressed as the percentage of the contraction induced by a supramaximal concentration of carbachol (10^{–5} M). In other experiments we studied the effects of (D-Pro², D-Trp^{7,9})-SP (10^{–3} M), atropine (10^{–6} M), guanethidine (5 × 10^{–6} M), phenoxybenzamine (10^{–7} M), propranolol (10^{–6} M), leu-enkephalin (10^{–9}–10^{–6} M) and tetrodotoxin (TTX) (10^{–6} M) on the responses to SP and to electrical stimulation. In a third series of experiments the effect of SP on the response to electrical field

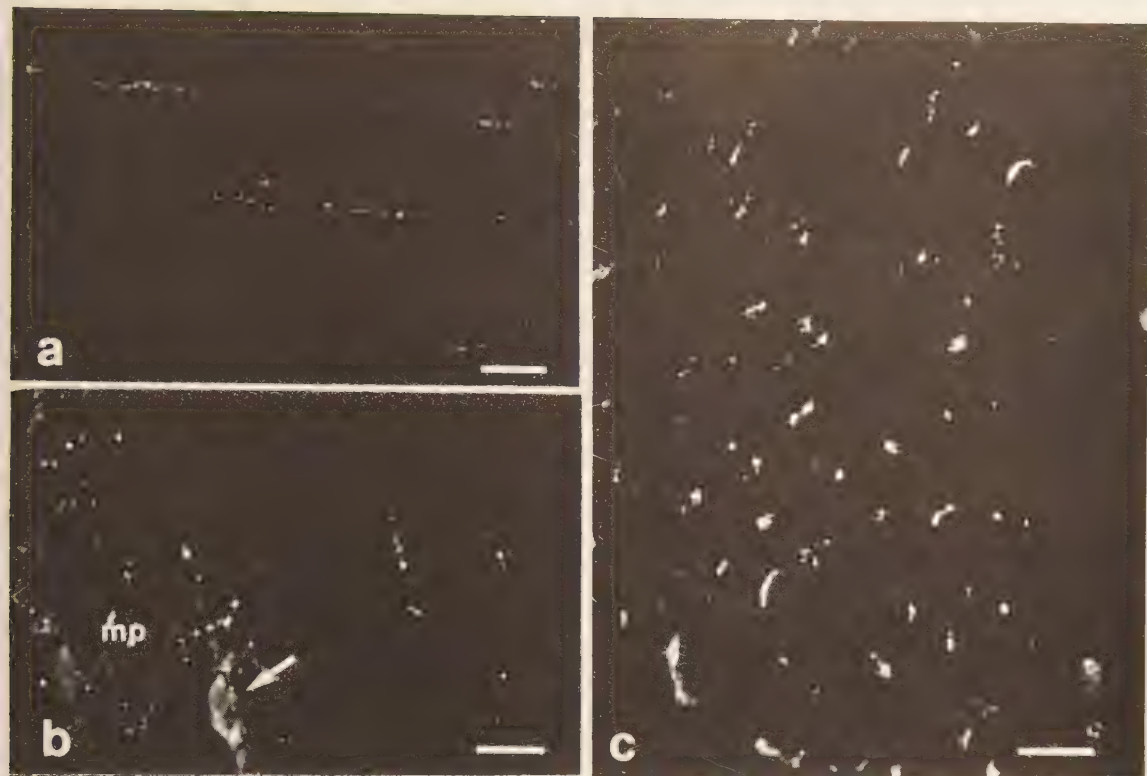


Fig. 1. Lower esophagus of cat. (a) Section of the longitudinal muscle layer. Fine varicose SP immunofluorescent fibres among the muscle bundles. (b) Section through myenteric plexus (mp) and circular muscle layer. Nerve cell body (arrow) displaying SP immunofluorescence together with numerous SP fibres. Fine varicose nerve fibres in the muscle layer outside the plexus. (c) Circular muscle layer. Numerous obliquely cut SP fibres. Bar indicates 50 μ m.

stimulation was studied. After the experiments the muscle strips were fixed in Bouin solution, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin for examination of the presence of striated and smooth muscle.

The following drugs were used: atropine sulphate (Vitrum, Stockholm, Sweden), carbamoylcholine (carbachol) (Merck, Darmstadt, FRG), guanethidine (Ciba-Geigy, Basel, Switzerland), phenoxybenzamine (Smith, Kline & French, Welwyn Garden City, England), propranolol (Imperial Chemical Industries, Macclesfield, England), synthetic bovine substance P and leu-enkephalin (Peninsula, San Carlos, Ca, USA), naloxone chloride (Endo, Garden City, N.Y., USA), tetrodotoxin (Sankyo, Tokyo, Japan). (D-Pro², D-Trp⁷⁻⁹)-SP, an SP antagonist (Folkers et al. 1981, Leander et al. 1981, Rosell et al. 1982), was generously supplied by Professor Karl Folkers, University of Texas, Austin, Texas.

RESULTS

Distribution of SP-immunoreactive nerve fibres

Fine varicose nerve fibres were observed in the esophageal smooth muscle layers of all species

studied (Fig. 1, 2). Of the two antisera tested one cross-reacted with bombesin. Both showed the same distribution and frequency of immunoreactive nerve fibres suggesting that the immunostaining reflected the presence of SP (or physalaemin). Immunoreactive nerve fibres were fairly numerous in the guinea-pig and cat but few in the pig. They were more numerous in the circular than in the longitudinal smooth muscle and the muscularis mucosae. Immunoreactive fibres did not seem to be associated with the striated muscle that predominated in the proximal parts of the esophagus. Striated muscle was found in the upper 2/3 of the esophagus in the cat and in the entire esophagus but for a narrow ring distally, in the other species. SP-immunoreactive fibres were numerous in the myenteric and submucosal plexuses, often surrounding nerve cell bodies that did not display SP immunoreactivity (Fig. 2). No conspicuous accumulation of fibres was noted in the region of the lower esophageal sphincter. SP-immunoreactive nerve fibres were not seen

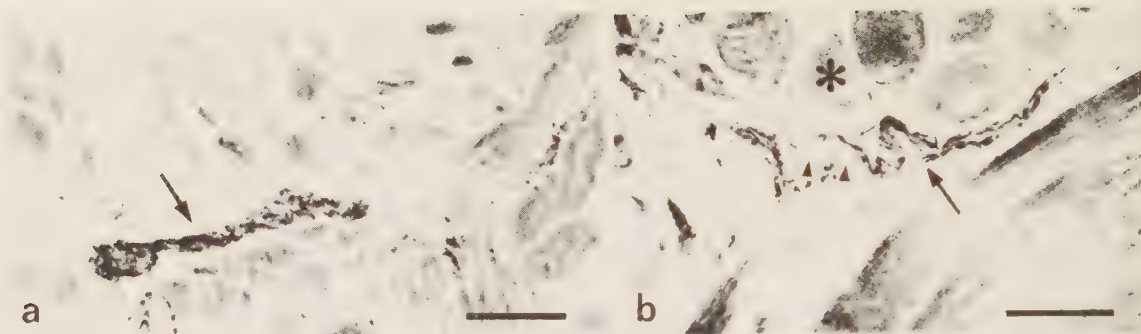


Fig. 2. Guinea pig esophagus, lower portion. (a and b) Numerous, fine varicose SP-immunoreactive nerve fibres in the myenteric plexus (arrows). In b large non-immunoreactive cell bodies (arrow-heads) are surrounded by SP fibres. SP fibres are by comparison much fewer in the smooth muscle. Note striated muscle (asterisk) close to the myenteric plexus in b. PAP staining. Bar indicates 50 μ m.

in the epithelium. SP fibres occurred around small blood vessels along the entire esophagus including those in striated muscle. Only in the cat were SP-immunoreactive nerve cell bodies detected. Such cell bodies were few and restricted to the myenteric plexus (Fig. 1).

Quantitation and analysis of immunoreactive SP

The lower part of the cat esophagus contained approximately 10 times more immunoreactive SP than the upper part, while the middle part contained intermediate amounts (Table 1). In all regions examined, the muscle layer of the esophageal wall contained more SP than the mucosa. The slopes of the dilution curves prepared with the tissue extracts, and also with physalaemin (not shown in

figure), were parallel to that of the SP standard curve (Fig. 3). The relatively low cross-reactivity of the antiserum with physalaemin supports the assumption that the immunoreactive material is identical with SP.

Motor activity of esophageal smooth muscle

The muscle strips were not spontaneously active. Upon electrical stimulation (1–5 Hz, 3 s) they usually responded with a biphasic contraction (Fig. 4). The first contraction, which was inconsistent and variable, occurred about 1 s after the start of the

Table 1. Concentration of extractable SP-like immunoreactivity in different segments of the cat esophagus

The concentrations are expressed relative to synthetic bovine SP. Values are given in pmol/g tissue wet weight $\pm$ SE (n=4–5)

Upper esophagus	
Muscle	1.03 $\pm$ 0.14
Mucosa	0.83 $\pm$ 0.15
Middle esophagus	
Muscle	6.43 $\pm$ 0.55
Mucosa	1.82 $\pm$ 0.30
Lower esophagus	
Muscle	9.59 $\pm$ 0.69
Mucosa	5.71 $\pm$ 0.98

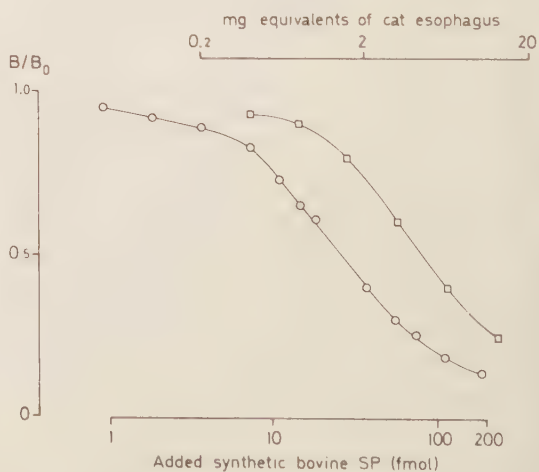


Fig. 3. Ratio of antibody bound tracer (B) to the maximally bound tracer in the absence of unlabelled SP (B_0) as a function of the added amount of synthetic SP (○—○) and tissue extracts (□—□).

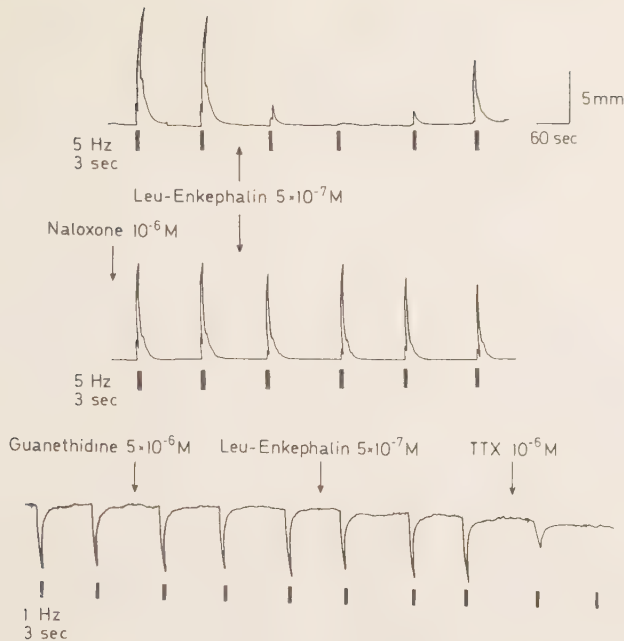


Fig. 4. The upper panel is a typical registration showing the inhibitory effect of leu-enkephalin on electrically evoked contractions of the circular muscle of the cat esophagus in vitro. The effect of leu-enkephalin lasts for about 5 min whereafter contractions reappear. The middle panel shows that the effect of leu-enkephalin is abolished by naloxone. The lower panel is a typical registration showing relaxatory responses to electrical stimulation in the circular muscle of cat esophagus. The relaxations are unaffected by adrenergic blockade (guanethidine) or leu-enkephalin but abolished by the neuronal blocker tetrodotoxin (TTX). Electrical stimulation and addition of drugs are indicated.

stimulation. Following this initial contraction the muscular tone was reduced. Immediately after cessation of stimulation, a second contraction was noted (see also Uddman et al. 1980). During the subsequent relaxation a small twitch was sometimes observed. In about 10% of the preparations the response to electrical stimulation was a relaxation instead of a contraction (Fig. 4). The contractile response to electrical stimulation was reduced by 10–15% (not statistically significant) by the α -adrenoceptor antagonist phenoxybenzamine and by the adrenergic blocker guanethidine while being unaffected by the β -receptor antagonist propranolol (not shown). The contractions could be completely blocked by TTX (not shown) which blocks nervous conduction in general (Kao 1966). It was also blocked by leu-enkephalin (Fig. 4) and atropine (Fig. 5) but not by the SP antagonist (D-Pro², D-Trp⁷⁻⁹)-SP (Fig. 5). The inhibitory effect of leu-enkephalin was blocked by the opiate receptor

antagonist naloxone (Fig. 4). The relaxation was not affected by adrenergic blockade, cholinergic blockade or by leu-enkephalin but was inhibited by TTX (Fig. 4). Carbachol and SP gave a concentration-dependent contractile response (Fig. 5, 6) in smooth muscle strips taken close to the esophago-gastric junction as well as in those obtained from more oral locations. These contractile responses could be blocked by atropine and (D-Pro², D-Trp⁷⁻⁹)-SP, respectively, but not by TTX. Addition of SP to the bath evoked an irregular spontaneous motor activity (Fig. 5). Atropine, guanethidine, phenoxybenzamine or propranolol affected neither the contraction nor the spontaneous motor activity induced by SP (not shown). In concentrations (10^{-8} – 10^{-7} M) that caused a slight increase in basal tension, SP greatly enhanced the contractile response to electrical nerve stimulation (Fig. 5). Also the myogenic responses obtained upon high frequency electrical stimulation (30 Hz, 10 s, 1 ms) of TTX-treated

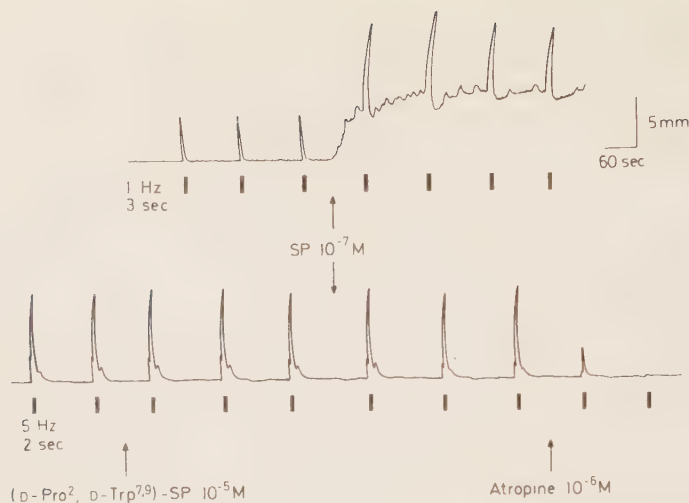


Fig. 5. A typical registration showing the increase in both tension and amplitude of electrically evoked contractions upon addition of substance P (SP) to the circular muscle of cat esophagus (upper panel). The effects of SP are prevented by the SP antagonist (D-Pro², D-Trp^{7,9})-SP and the contractions are blocked by atropine (lower panel). Electrical stimulation and addition of drugs are indicated.

preparations were enhanced by SP. Both the enhancement of the contractile response to low frequency electrical stimulation of control preparations and the enhancement of myogenic responses to high frequency electrical stimulation of TTX-treated preparations were counteracted by (D-Pro², D-Trp^{7,9})-SP.

DISCUSSION

Nerve fibres displaying SP immunoreactivity were present in the esophagus of all the species examined. Fine varicose nerve fibres were seen in the circular muscle and to a smaller extent in the longitudinal muscle and muscularis mucosae. In the myenteric and submucosal plexuses immunoreactive fibres were found to ramify around nerve cell bodies not displaying SP immunoreactivity. Occasionally, a few SP-immunoreactive nerve cell bodies were encountered in the myenteric plexus of the cat esophagus. In addition, SP-containing nerve fibres were seen around small blood vessels in the entire esophagus.

Recently a physalaemin-like substance was demonstrated in various mammalian tissues (Lazarus et al. 1980). Physalaemin has the C-terminal tripeptide and a phenylalanine residue in position 7 in common with SP. At the dilutions used in immunocyto-

chemistry, all SP antisera used in the present study cross-react with physalaemin. It is therefore possible that physalaemin may have contributed to the immunoreactivity detected in the esophageal nerve fibres. On the other hand, significant interference of this peptide with the radioimmunochemical determination of SP is unlikely, since the cross-reactivity of antiserum K 25 with physalaemin in the radioimmunochemical procedure is less than 5% (Nilsson et al. 1977, own unpublished observations) and since the tissue levels of physalaemin-like peptide reported by Lazarus et al. (1980) were lower than those of SP-like immunoreactivity (Brodin & Nilsson 1981).

In view of the potent vasodilatory actions of SP cf. Hallberg & Pernow 1975) it is conceivable that SP takes part in the regulation of local blood flow in the esophagus. In addition, SP may be involved in the functional control of non-vascular smooth muscle. We have previously observed a rich distribution of nerve fibres containing VIP or enkephalin in the esophageal smooth muscle (Uddman et al. 1978, 1980). VIP fibres were particularly numerous in the esophago-gastric junction, suggesting a role for VIP in the regulation of sphincter tone (Uddman et al. 1978). Neither enkephalin fibres (Uddman et al. 1980) nor SP fibres were conspicuously accumulated in the sphincter region.

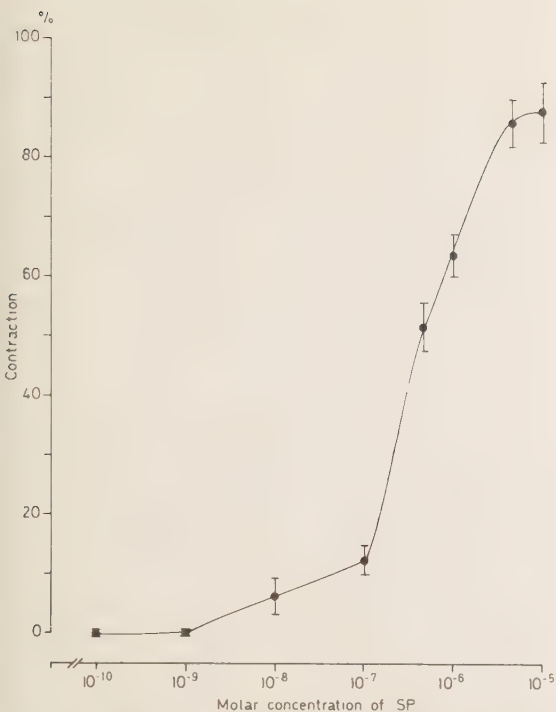


Fig. 6. Concentration-response curve showing the effect of SP on the tension of the circular muscle of the cat esophagus. Ordinate: Increase in tension as a percentage of the contraction induced by carbachol (10^{-5} M). Abscissa: SP concentration (M). Each value is the mean of 4-16 experiments. Vertical bars give SE.

Relatively low concentrations of immunoreactive SP have previously been found in the esophagus of dog and rat (Nilsson & Brodin 1977, Holzer et al. 1980, Brodin & Nilsson 1981) compared to the values now reported for the cat. Even though different extraction procedures have been used, it seems obvious that the concentration of immunoreactive SP in the esophagus differs greatly between species. In the dog and rat the lower part of the esophagus consists mostly of striated muscle, whereas in the cat smooth muscle predominates. The latter species was therefore chosen for the *in vitro* experiments. It is notable that in the cat esophagus the concentrations of immunoreactive SP were approximately 10 times higher in the lower part than in the upper part. This observation is in good agreement with the immunocytochemical findings. The circular smooth muscle of the feline lower esophagus regularly responded to electrical stimulation with a biphasic contraction. The contractions were reduced somewhat by α -adrenergic receptor

blockade; the remaining contraction was completely abolished by muscarinic receptor blockade suggesting the existence of a cholinergic pathway in esophageal motor function (Fournet et al. 1980). In this respect our data differ from those obtained by Lund & Christensen (1969) on opossum esophagus and by Uddman et al. (1980) on cat esophagus, since in both these studies atropine had marginal effects only on the contractile response to electrical stimulation. Leu-enkephalin inhibited the electrically induced contractile responses in a concentration-dependent manner. This effect was blocked by naloxone, suggesting the involvement not only of muscarinic and α -adrenergic receptors in the contractile response to electrical stimulation but also of opiate receptors. Conceivably, the inhibitory effect of leu-enkephalin is exerted on cholinergic fibres. SP elicited a concentration-dependent contraction that was unaffected by α -adrenergic and cholinergic blockade and by TTX. A specific SP antagonist, (D-Pro², D-Trp⁷⁻⁹)-SP (Folkers et al. 1981, Leander et al. 1981, Rosell et al. 1982), blocked the SP-induced contraction but had no effect on the response to electrical stimulation. The presence of receptors for SP on the smooth muscle cells is interesting in view of the existence of SP-containing nerve fibres in the esophageal smooth muscle. However, the experiments with the SP antagonist provided no evidence that SP is directly involved in esophageal neuromuscular transmission. On the other hand, SP enhanced the motor response to electrical stimulation. This enhancement may be exerted by an action directly on the muscle cells or via presynaptic potentiation of cholinergic transmission.

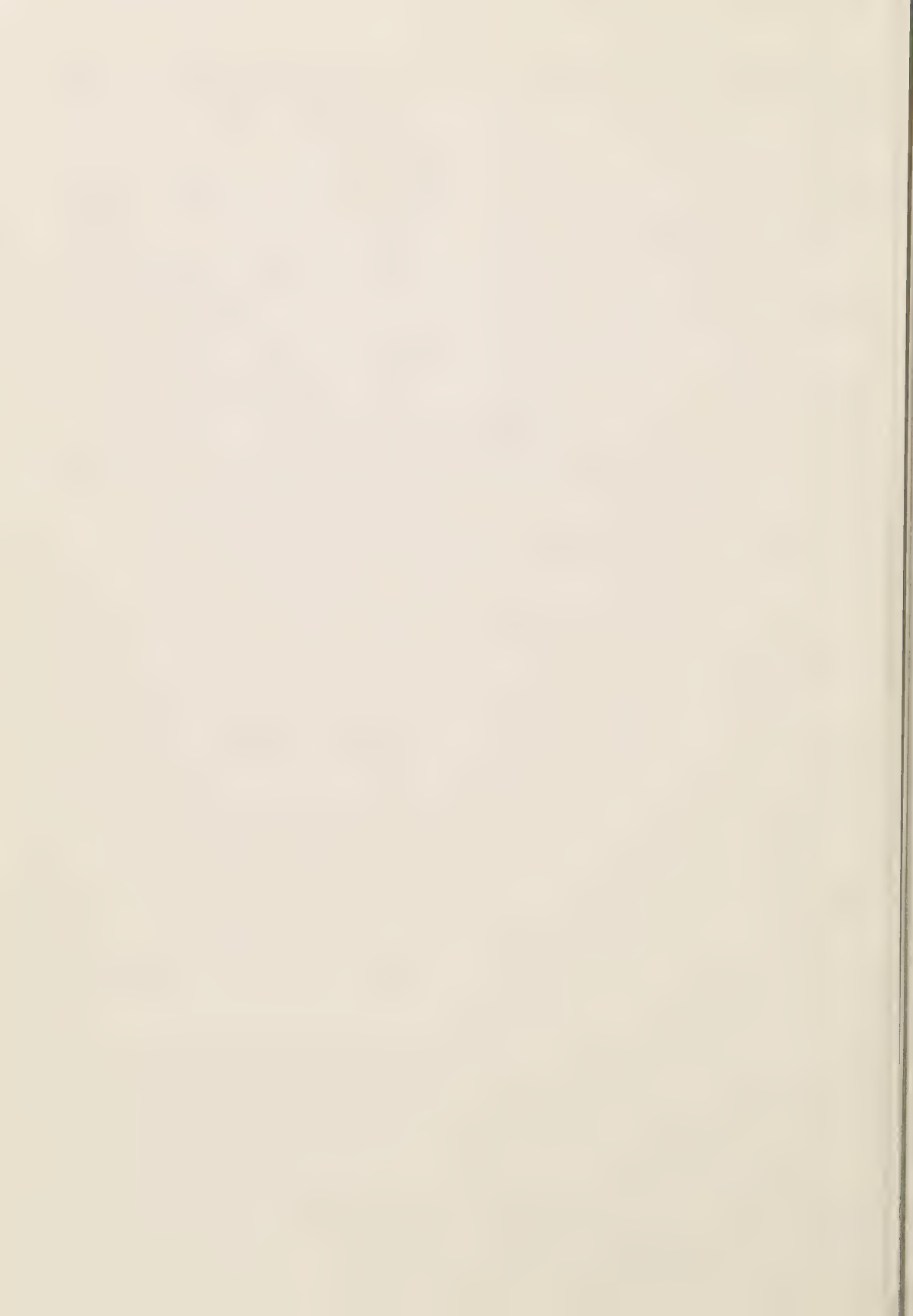
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Substance P-immunoreactive nerve fibres in the anterior segment of the rabbit eye

Distribution and possible physiological significance

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Summary. Substance P-immunoreactive nerve terminals were found in several locations in the anterior segment of the rabbit eye. In the iris they occurred in the sphincter muscle and were randomly distributed in the iris stroma with some fibres running close to the dilator muscle. In the ciliary body these immunoreactive elements were few and occurred within bundles of nerve fibres, while in the ciliary processes they were more numerous with a predominantly subepithelial location. Blood vessels in the anterior uvea were often surrounded by substance P-immunoreactive fibres. No substance P-fibres were found in the cornea, while the sclera contained very few such elements.

Using conventional *in vitro* techniques it was found that the sphincter pupillae muscle of the iris responded to electrical stimulation with a contraction that was resistant to cholinergic and adrenergic blockade, but was inhibited by the neuronal blocker tetrodotoxin. This indicates the existence of a non-cholinergic, non-adrenergic neuronal mediator of the contractile response. Exogenously applied substance P produced a long-lasting contraction of the spincter muscle, an observation compatible with the view that substance P is the noncholinergic, non-adrenergic neurotransmitter involved.

Key words: Neuropeptides – Substance P – Uvea Immunocytochemistry – Iris – Smooth muscle contraction – Rabbit

Substance P (SP) is a neuropeptide with a widespread distribution in the central nervous system (e.g., Hökfelt et al. 1977; Ljungdahl et al. 1978), including the retina (Karten and Brecha 1980), and in the peripheral nervous system (e.g., Nilsson et al. 1975; Hökfelt et al. 1977; Alm et al. 1978). SP occurs in primary sensory neurones (Hökfelt et al. 1975a, b), and it has been suggested that at least a proportion of the peripheral SP-containing fibres represent the dendritic ramifications of such neurones. It has also been suggested that SP is a causative factor in the “irritative”

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response to antidromic nerve stimulation and consequently involved in "axon reflexes" (Lembeck and Holzer 1979). This hypothesis is supported by the finding that SP-immunoreactive material is released from the tooth pulp upon electrical stimulation of the mandibular nerve (Olgart et al. 1977). The presence of SP in the anterior uvea of the rabbit is suggested by the results of radioimmunoassay (Butler et al. 1980; Camras and Bito 1980). Electrical or mechanical stimulation of the trigeminal nerve causes an increase in the amount of SP-like material in the aqueous humour, concomitant with miosis, vasodilation, breakdown of the blood-aqueous barrier and a rise in the intraocular pressure (Bill et al. 1979). In the same species intracameral injection of SP mimics these effects (Bill et al. 1979). Surgical denervation as well as treatment with capsaicin markedly reduces the SP concentration in the anterior uvea and modifies the reaction to irritative substances such as prostaglandin E_1 , nitrogen mustard, bradykinin and capsaicin, but not the reaction to SP (Butler and Hammond 1980; Camras and Bito 1980). Together these results suggest that SP is one of the substances released by antidromic nerve stimulation to act as a mediator of irritative responses.

The present report deals with the distribution of SP-immunoreactive nerve fibres in the anterior segment of the rabbit eye and the possible functional significance of SP fibres in the iris.

Materials and methods

Immunocytochemistry

Eight albino or pigmented rabbits weighing 1–2 kg were used. Six rabbits were anaesthetized with Brialat® and perfused through the ascending aorta with 1 l of an ice-cold 4% formalin solution (40 g paraformaldehyde in 1 l 0.1 M phosphate buffer; pH adjusted to 7.0 with 1 N NaOH). The anterior uvea was rapidly dissected out and stored in the fixative for 24–48 h and rinsed for 24 h in ice-cold Tyrode buffer containing 5% sucrose. The specimens were frozen on dry ice and sectioned in a cryostat at -15°C . Two rabbits were killed by i.v. injection of air. The irides were rapidly dissected out and placed as whole mounts on glass slides. The pigment layer was removed by gentle scraping with a scalpel. The preparations were fixed by immersion in buffered picric acid-formaldehyde solution overnight. They were then dehydrated in graded ethanol solutions, cleared in xylene and rehydrated (cf. Furness et al. 1980). The cryostat sections (15 μm) and whole-mount preparations were processed for the immunocytochemical demonstration of SP using the indirect immunofluorescence technique (Coons et al. 1955). Each section was exposed to SP antiserum in the appropriate dilution for 3 h at room temperature in a moisture chamber. After thorough rinsing in phosphate-buffered saline, pH 7.2, the site of the antigen-antibody reaction was revealed with fluorescein isothiocyanate labelled sheep anti-rabbit Ig G (SBL, Stockholm, Sweden), used in dilution 1 : 20. Incubation was for 30 min at room temperature. The antisera contained 0.25% serum albumin and 0.25% Triton X-100. For details of the SP antisera used, see Table 1. Immunofluorescence was observed in a fluorescence microscope with filters selected to give peak excitation at 490 nm. Controls were as follows: 1) the SP antiserum was omitted; 2) the second (fluoresceinated) antibody was omitted; 3) normal rabbit antiserum was applied instead of SP antiserum; 4) each SP antiserum was permitted to react with synthetic SP (10–100 μg ml dilute antiserum) for at least 24 h before being applied to the sections. Such absorption of the antisera resulted in abolishment of the immunoreaction.

For reasons of convenience and simplicity we refer to the immunoreactive material as SP although it must be realized that the antibodies will detect also other peptides or proteins that share the immunogenic amino-acid sequence with SP (see Table 1).

Motor activity studies

The functional properties of the isolated iris sphincter pupillae muscle were studied using conventional *in vitro* techniques. The muscles were excised from 12 pigmented rabbits (1–2 kg), opened and mounted

Table 1. Characteristics of SP antisera

Code no	Working dilution (immunofluorescence)	Source
JK 1	1:320	Dr. P.C. Emson, Dept. of Pharmacology, Medical School, Cambridge, England
SP 8	1:80	Dr. P.C. Emson, Dept. of Pharmacology, Medical School, Cambridge, England
L-83	1:40	Dr. G. Dockray, Dept. Physiol, Univ. Liverpool, England
R 2404	1:40	Dr. N. Yanaihara, Shizuoka Med. Coll., Japan
24259	1:160	Immunonuclear Corporation, Stillwater, Minnesota, USA

All the antisera appear to be directed against the carboxy-terminal end since they were inactivated by absorption with physalaemin and eledoisin (Peninsula, Calif., USA) in concentrations of 100 µg per ml diluted antiserum (Brodin et al. 1981). Antibodies directed against the carboxy-terminal tri-peptide end of SP will detect other peptides sharing this sequence, e.g., the tachykinins, which include physalaemin and eledoisin (Brodin et al. 1981). Neither of the antisera cross-react with bombesin at the immunocytochemical level. Although the immunoreactive material can be identified as true SP by chemical analysis only, we refer to the immunoreactive fibres as SP fibres for reasons of simplicity

in organ baths at 35–36°C, and connected to isotonic transducers for registration of mechanical activity. The load on the muscle was 0.2 g, and the bathing fluid had the following composition (in mM): NaCl 133; NaHCO₃ 16.3; KCl 4.7; MgCl₂ 1.0; NaH₂PO₄ 1.4; CaCl₂ 2.5; and glucose 7.8. The muscles were allowed to equilibrate in the bath for 60 min before experimentation started. Platinum ring electrodes were placed around the muscles with a constant electrode distance of 5 mm. The electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (15–50 V over the electrodes; 1–5 ms duration). The muscles were stimulated with trains of pulses lasting for 10 s and with a frequency of 10–30 Hz. The mechanical activity of the preparation was recorded continuously throughout the experiment on a Grass model 7 Polygraph.

Synthetic SP was obtained from Beckman, Geneva, Switzerland; tetrodotoxin from Sankyo, Tokyo, Japan; phenoxybenzamine from Smith, Kline and French, Welwyn Garden City, England; propranolol from Imperial Chemical Industries, Macclesfield, England; guanethidine from Ciba-Geigy, Basle, Switzerland; and atropine sulphate from ACO, Stockholm, Sweden.

Results

Distribution of SP-immunoreactive fibres

SP-immunoreactive nerve terminals were found in varying numbers in several parts of the anterior uvea. The immunocytochemical findings are summarized in Fig. 1. Generally, the fibres were thin and beaded with stronger fluorescence in the varicosities than in the intervaricose parts.

In the iris, immunoreactive fibres were seen to run immediately in front of the dilator muscle, within the sphincter muscle (Fig. 2) and close to blood vessels (Fig. 3). The SP fibres did not seem to penetrate into the dilator muscle. In the sphincter muscle they were most numerous in the part opposing the pupillary margin (Figs. 1, 2). The iris stroma contained a moderate number of SP fibres forming a loose plexus (Fig. 4). They had no obvious association to any specific structure within the stroma and were too few to permit evaluation of differences in

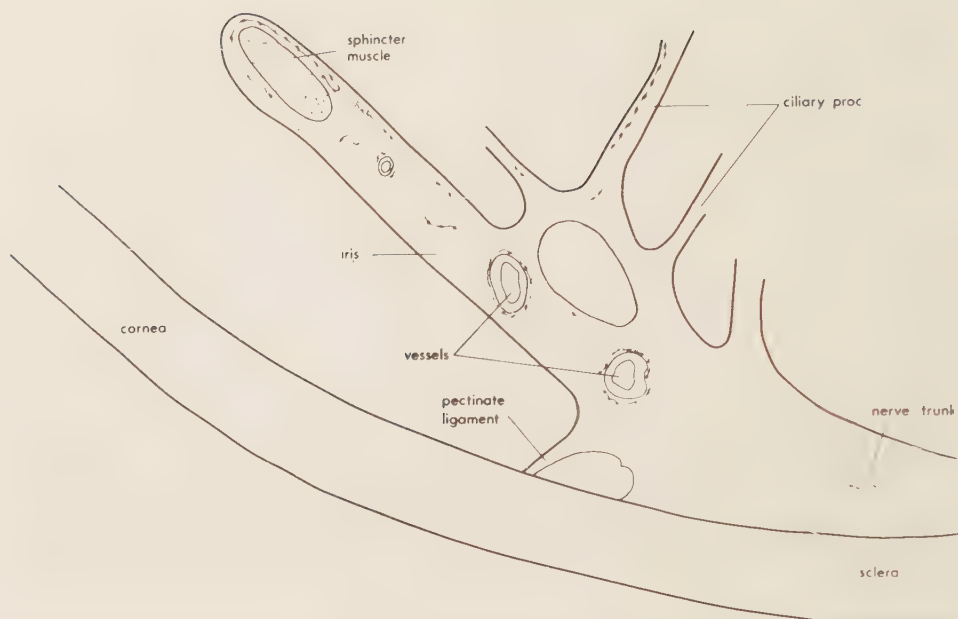


Fig. 1. Schematic drawing of the distribution of substance P-immunoreactive nerve fibres in the anterior segment of the rabbit eye. Immunoreactive fibres are found predominantly in the sphincter muscle, randomly distributed in the iris stroma, adjacent to blood vessels, in nerve trunks of the ciliary body, and in the ciliary processes

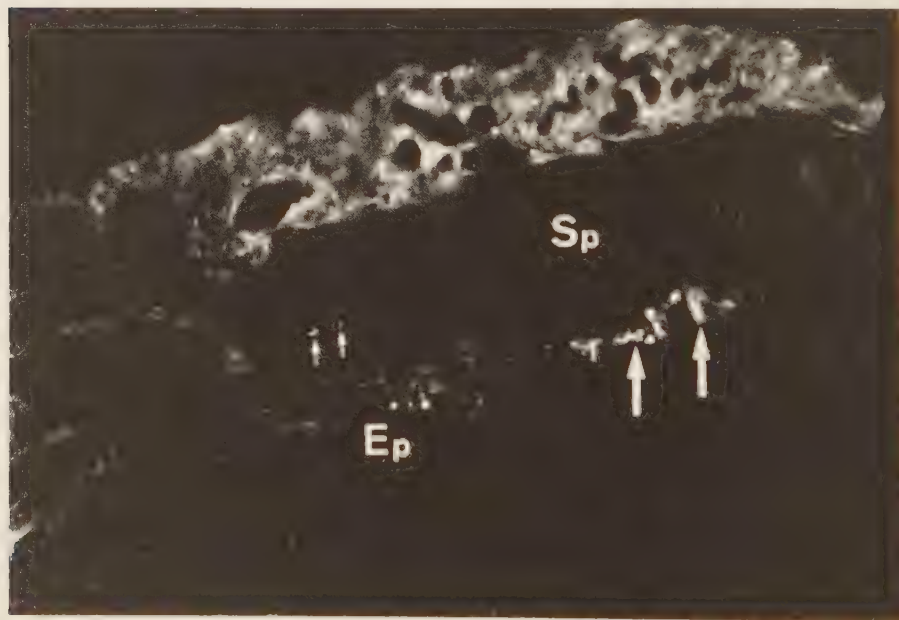


Fig. 2. Substance P-immunofluorescent fibres in the iris. They are found within the sphincter muscle, notably in the part opposing the pupillary margin (*small arrows*), but they also occur in the vicinity of the muscle (*large arrows*). Note autofluorescence in the stroma. *Ep* epithelium on the posterior iris surface; *Sp* sphincter. $\times 280$



Fig. 3. Substance P-immunofluorescent nerve fibres (*arrowheads*) close to a blood vessel in the iris. Smaller vessels lack immunoreactive nerve fibres. *Ep* epithelium on the posterior iris surface. $\times 310$



Fig. 4. Whole mount of rabbit iris. Substance P-immunofluorescence in nerve fibres forming a loose plexus in the stroma. $\times 250$



Fig. 5. **a** Substance P-immunofluorescent nerve fibres in anterior ciliary processes. These fibres can occasionally be followed over long distances. **b** Substance P-immunoreactive nerve fibre (*arrowheads*) in an anterior ciliary process. The fibre is not strictly allied with the blood vessel (*V*) but seems to be interposed between the epithelium and the blood vessel. $\times 280$

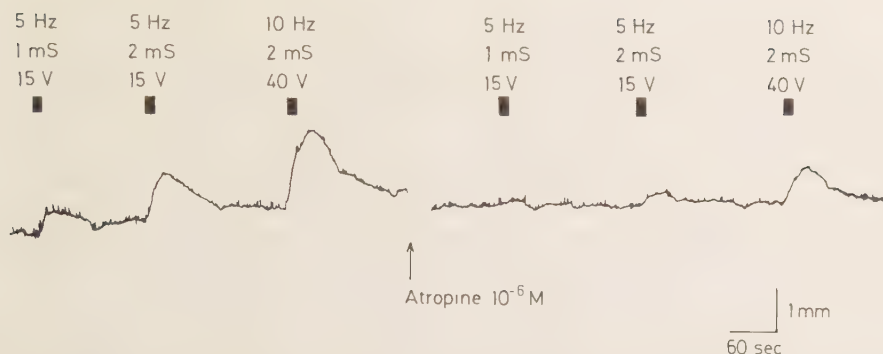


Fig. 6. Registrations showing the amplitude of responses to different stimulation conditions of the rabbit sphincter pupillae muscle before (*left*) and after (*right*) atropine. Electric stimulation of 10 s is indicated. Note the presence of an atropine-resistant contractile response

fibre frequency within the stroma. Bundles of nerve fibres running in the stroma often contained a few varicose SP fibres. SP fibres were more numerous around the larger blood vessels than around smaller vessels.

Immunoreactive fibres were rare in the trabecular tissue of the chamber angle; only occasionally were short SP fibres seen near the aqueous channels in the sclera.

The ciliary body contained very few SP fibres, without obvious relation to the ciliary muscle. Occasionally, they occurred within nerve fibre bundles together with non-immunoreactive fibres. The anterior ciliary processes often contained varicose SP fibres, which at times could be followed over long distances (Fig. 5a). They had a subepithelial location, often interposed between blood vessels and the epithelial lining (Fig. 5b). The posterior ciliary processes contained SP fibres infrequently.

The cornea displayed a disturbingly high background fluorescence (auto-fluorescence and unspecific binding of antiserum); no SP fibres could be demonstrated in the cornea. The sclera contained extremely few fibres. All antisera gave identical results.

Motor activity of the iris

Upon electrical stimulation the sphincter pupillae muscle responded with a long-lasting contraction. Addition of the cholinergic blocker atropine caused a reduction in the contractile response to low frequency stimulation, while responses to stimulation with higher frequency or longer pulse duration were less affected (Fig. 6). Addition of adrenergic alpha- and beta-blockers (phenoxybenzamine and propranolol) or guanethidin (5×10^{-6} M; not shown in Fig.) failed to inhibit the contractile response (Fig. 7). Addition of the neuronal blocker tetrodotoxin (TTX) (Kao 1966) abolished all responses to electrical stimulation. SP in concentrations above 10^{-9} M gave a long-lasting contraction of the sphincter muscle (Fig. 7). This response was not affected by cholinergic or adrenergic blocking agents or by TTX.



Fig. 7. Registration showing the effect of cholinergic (atropine), adrenergic (phenoxybenzamine and propranolol) receptor blockade, and of tetrodotoxin (TTX) on electrically evoked responses in the sphincter pupillae muscle. The muscles were stimulated when indicated with 30 Hz, 5 ms and 30 V over the electrodes. Each train of pulses lasted for 10 s. Note that the contraction persisted after cholinergic and adrenergic receptor blockade, while the neuronal blocker TTX abolished all contractile responses. As a final step in this sequence SP was added to the bath

Discussion

The number of SP-immunoreactive fibres in the uvea as a whole was small compared with that of adrenergic and acetylcholinesterase-positive fibres (Ehinger 1964, 1966), although they showed a similar distribution. Generally, the SP fibres were associated with smooth muscle or with the ciliary processes. In the iris SP fibres were found in the sphincter muscle as well as in front of the dilator muscle. In the sphincter muscle the SP fibres, like the adrenergic fibres (Ehinger 1964, 1966), were most numerous in the part opposing the pupillary margin. In this region the number of SP fibres was similar to that of adrenergic fibres. This was the case also in the ciliary processes. Tervo et al. (1980a, b), using monoclonal antibodies, recently reported their findings of SP fibres in the uvea of the guinea-pig and rabbit. On the whole, the distribution described was similar to that reported here. However, unlike Tervo et al. (1980a, b), we did not detect SP fibres in the cornea. This discrepancy in results may be due to the different histotechnical processing and/or different antisera used. The absence or paucity (Tervo et al. 1980a, b) of SP fibres in the cornea is in contrast to the rich number of sensory fibres previously demonstrated by Weddel and Zander (1950). Thus it is probable that nociception in the cornea does not depend solely upon SP.

In vivo experiments have demonstrated that SP is a potent miotic agent (Mandahl and Bill 1980a; Masuda et al. 1980; Stjernschantz et al. 1981). It causes contraction of the sphincter pupillae muscle at a dose level of 0.2–1 ng injected into the anterior chamber (Mandahl and Bill 1980a). With higher doses there is vasodilation, breakdown of the blood-aqueous barrier and a rise in intraocular pressure (Bill et al. 1979). Prompt miosis and release of SP-like immunoreactivity is produced by electrical or mechanical stimulation of the trigeminal nerve, indicating that at least a proportion of the SP fibres are branches from this nerve (Bill et al. 1979). The miosis produced by trigeminal nerve stimulation is notably long-lasting, unlike that resulting from stimulation of the oculomotor nerve (Perkins 1957). In vitro the iris sphincter pupillae muscle of the rabbit responded to SP or electrical stimulation with contraction. The response to electrical stimulation was unaffected by adrenergic blockade, reduced but not abolished by cholinergic blockade, and completely abolished by tetrodotoxin, which blocks nervous conduction in general (Kao 1966). As shown in Fig. 7 the contractile effect of SP was not affected by adrenergic or cholinergic blockers or by TTX. The effect of atropine was less pronounced when the muscles were stimulated with high frequency and long pulse duration (cf. Figs. 6, 7). Conceivably, these stimulation conditions lead to more efficient release of the non-cholinergic neurotransmitter responsible for the contraction. Hence, the present results suggest that the nerve fibres involved have a higher stimulation threshold than the cholinergic fibres. The present findings in vitro are in good agreement with those previously obtained in vivo (Mandahl and Bill 1980a, b; Mandahl and Bill 1981; Masuda et al. 1980; Stjernschantz et al. 1981) as well as in vitro (Soloway et al. 1981). Together, they support the existence of a neuronal, non-cholinergic, non-adrenergic mechanism for the contraction of the iris sphincter pupillae muscle. They also support the view that SP is the excitatory transmitter involved.

In the iris, SP fibres were in close contact with the blood vessels. Interestingly, both SP and trigeminal nerve stimulation cause vasodilatation in the iris (Bill et al. 1979; Mandahl and Bill 1981). In the ciliary processes the SP fibres run close to both blood vessels and the epithelium. However, they are not strictly associated with the blood vessels. They may therefore be functionally related to the epithelium rather than to the blood vessels. There are no data available on the influence of SP on the function of the ciliary epithelium.

Together, the observations suggest that SP nerve fibres in the anterior segment of the eye originate from the trigeminal nerve, and that neuronal SP is of physiological significance in functions of the uvea. In the present study the SP fibres had the beaded appearance typical of axonal terminals. However, it cannot be excluded that the SP fibres are sensory; consequently the terminals might be regarded as dendritic and not as axonal elements. The demonstration of SP-immunoreactive nerve fibres in the uvea adds a fourth defined population of uveal nerves to the three previously known, i.e., adrenergic, cholinergic, and VIPergic (Uddman et al. 1981).

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Short title: Bradykinin releases neuronal SP

BRADYKININ CONTRACTS THE PUPILLARY SPHINCTER AND EVOKES OCULAR
INFLAMMATION THROUGH RELEASE OF NEURONAL SUBSTANCE P

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1 Bradykinin contracts the isolated rabbit sphincter pupillae muscle. The contraction was resistant to atropine but sensitive to tetrodotoxin, suggesting a noncholinergic nervous mechanism. The contraction was blocked by specific substance P (SP) antagonists suggesting the involvement of SP. The SP antagonists tested were ($\underline{\text{D}}\text{-Pro}^2$, $\underline{\text{D}}\text{-Trp}^{7,9}$)-SP and Arg-Gln- $\underline{\text{D}}\text{-Trp}$ -Phe- $\underline{\text{D}}\text{-Trp}$ -Leu-Met-NH₂.

2 The bradykinin-induced contraction exhibited marked tachyphylaxis in contrast to that induced by SP. The tachyphylaxis was associated with a reduction of the SP-mediated contractile response to electrical stimulation. Hence, it appears that the tachyphylaxis reflects the depletion of a bradykinin-sensitive neuronal pool of SP.

3 Injection of bradykinin into the vitreous chamber of the rabbit eye causes symptoms of inflammation, such as miosis and aqueous flare. Repeated administration of bradykinin a few hours after the first administration evoked a much reduced response; the response to SP was unchanged following repeated administration. Atropine was without effect on the response to bradykinin whereas tetrodotoxin and the SP antagonists abolished the response. The results suggest that bradykinin causes ocular inflammation through local release of neuronal SP.

Introduction

Bradykinin, a nonapeptide found in blood, is thought to be involved in the mediation of several pathophysiological conditions, including inflammation and pain generation (Eisen, 1970; Rocha e Silva, 1970; cf Erdös, 1979). It is widely accepted that like kinins in general bradykinin arises from the proteolytic degradation of plasma proteins. On the other hand, there is evidence that bradykinin is a neuronal constituent (Inouye et al., 1961;

Hori, 1968; Correa et al., 1979). Not only its site of origin but also its mechanism of action is unknown. Butler, Cole & Zhang (1981) recently reported that the bradykinin-induced contraction of the isolated rabbit sphincter pupillae muscle displayed tachyphylaxis and that trigeminal denervation prevented the contractile response (see also Butler & Hammond, 1980). They suggested that bradykinin acts by releasing a neurogenic mediator, possibly substance P (SP) (Butler et al., 1981). We have analyzed the mechanism of action of bradykinin with respect to its effect on the isolated rabbit iris sphincter pupillae and its role in inflammation of the eye. SP nerve fibres have previously been implicated in the noncholinergic neuronal contraction of the sphincter pupillae muscle (Leander et al., 1981) and SP is thought to be one of the mediators of ocular inflammation (Holmdahl et al., 1981).

Methods

The sphincter pupillae muscle of the rabbit iris was excised, opened and mounted vertically on a Perspex holder in a 7-ml organ bath maintained at 35 °C. One end was attached to a rigid support and the other to a lever connected via a spring to a Grass FT03 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. The load on the muscle was set at 0.2 g. Platinum ring electrodes were placed around the muscle with a constant electrode distance of 5 mm and the electrodes were connected to a Grass S 4C stimulator for field stimulation with square wave pulses (15 V over the electrodes, 0.5-1 ms duration). The muscles were stimulated with trains of pulses lasting 10 s and with a frequency of 5-20 Hz, the resting period between stimulations being at least 2 min. The contractile responses to bradykinin and SP were analyzed by the use of atropine, tetrodotoxin (TTX) and SP antagonists. TTX is a well-known blocker of nervous conduction (Kao, 1966). The SP antagonists were (D-Pro², D-Trp^{7,9})-SP (Holmdahl et al., 1981; Leander et al., 1981) and Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH₂ (Höriq et al., to be published). The mechanical activity of the preparation was continuously recorded on a Grass model 7 or model 5 polygraph. The bathing fluid was a modified Krebs solution of the following composition (mM): NaCl 133, NaHCO₃ 16.3, KCl 4.7, MgCl₂ 1.0, NaH₂PO₄ 1.4, CaCl₂ 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7% CO₂ in O₂ giving a pH of 7.2-7.3.

In another series of experiments we examined the effects of atropine, TTX and of the two SP antagonists on the response of the rabbit eye to intravitreal injections of bradykinin. Adult pigmented rabbits (1.5 to 3 kg) of mixed strain were anaesthetized with methohexital sodium (Brietal^(R), Lilly; 5 mg/kg) when given injections into the eye. No anaesthesia was ad-

ministered during the rest of the experiments. The time course of the barrier damage induced by intravitreal injection of bradykinin was followed by photo-electric measurement (Bengtsson, 1975) of the aqueous flare response (AFR). This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and the protein concentration has been established (Anjou & Krakau, 1961). The results were expressed in arbitrary units with reference to a standard (Anjou & Krakau, 1961). The AFR and pupillary diameter were measured every 30 min unless otherwise stated. Bradykinin (350 pmole in 15 μ l), SP (3 nmole in 9 μ l) or 0.9 percent saline (9 or 15 μ l) was injected into the corpus vitreum by means of Hamilton precision syringes, 3 to 4 mm posterior to the limbus. Atropine 1% and the SP antagonist Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH₂ were applied topically in a volume of 60 μ l to the left eye. Atropine was administered 1 hour before injection of bradykinin. TTX (15 nmole in 10 μ l) was injected intravitreally 4 hours before bradykinin. The SP antagonist was given twice, 1 hour and 10 min before bradykinin. If not otherwise stated, the right eye received 0.9 percent saline topically before injection of bradykinin.

Bradykinin and SP were purchased from Beckman, Geneva, Switzerland. The SP analogues were synthesized by techniques analogous to those described elsewhere (Folkers et al., 1981; Rosell et al., 1982). Their purity was tested by high performance liquid chromatography and found to be better than 98%. They have been identified as specific SP antagonists (Leander et al., 1981; Horig et al., to be published) and previously found not to block the motor response of isolated guinea pig taenia coli to bradykinin (Leander et al., 1981). TTX was obtained from Sankyo, Tokyo, Japan. Atropine sulfate was from Alcon, Fort Worth, Texas, USA.

Results

The addition of SP to the isolated sphincter pupillae muscle produced a slow contraction, reaching a maximum 120-150 s after addition to the bath (Fig. 1). The bradykinin-induced contraction was even slower in onset. Repeated administration of SP (10^{-8} M) produced the same response, whereas the response to repeated administration of bradykinin (10^{-8} - 10^{-6} M) was abolished, despite repeated washings (Fig. 1). After the sphincter pupillae muscle had been made insensitive to bradykinin it retained its capacity to respond to SP. The contractile response to bradykinin, but not that to SP, was abolished by pretreatment with TTX (Fig. 1). These results indicate a neuronal mediation of the response to bradykinin. The contractile responses to SP and bradykinin were greatly reduced or abolished by pretreatment

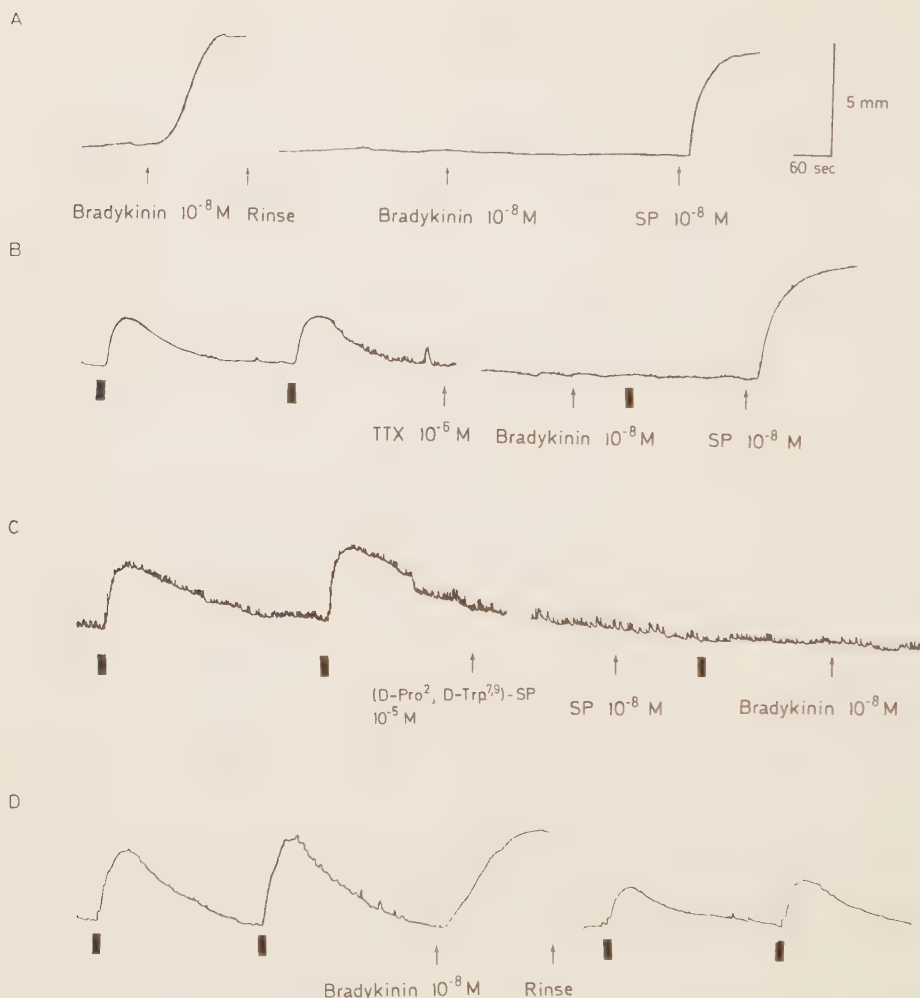


Fig. 1. Recordings showing typical results of in vitro experiments with the isolated rabbit iris sphincter pupillae muscle. Atropine (10^{-6} M) was present throughout. The muscles were stimulated electrically (10 Hz, 10 sec) (indicated by black rectangles). A. Contractions induced by bradykinin and SP. Note lack of effect of repeated application of bradykinin. B and C. Effects of TTX (B) and the SP-blocking agent (D-Pro², D-Trp^{7,9})-SP (C) on contractile responses to electrical stimulation, bradykinin, or SP. The bradykinin-induced contraction was inhibited by TTX and by the SP antagonist. The preparations were allowed to rest for about 10 min after addition of TTX or SP to the bath. D. Pretreatment with bradykinin greatly reduced the amplitude of the electrically evoked contractions. The preparation was not stimulated until the tension had returned to baseline.

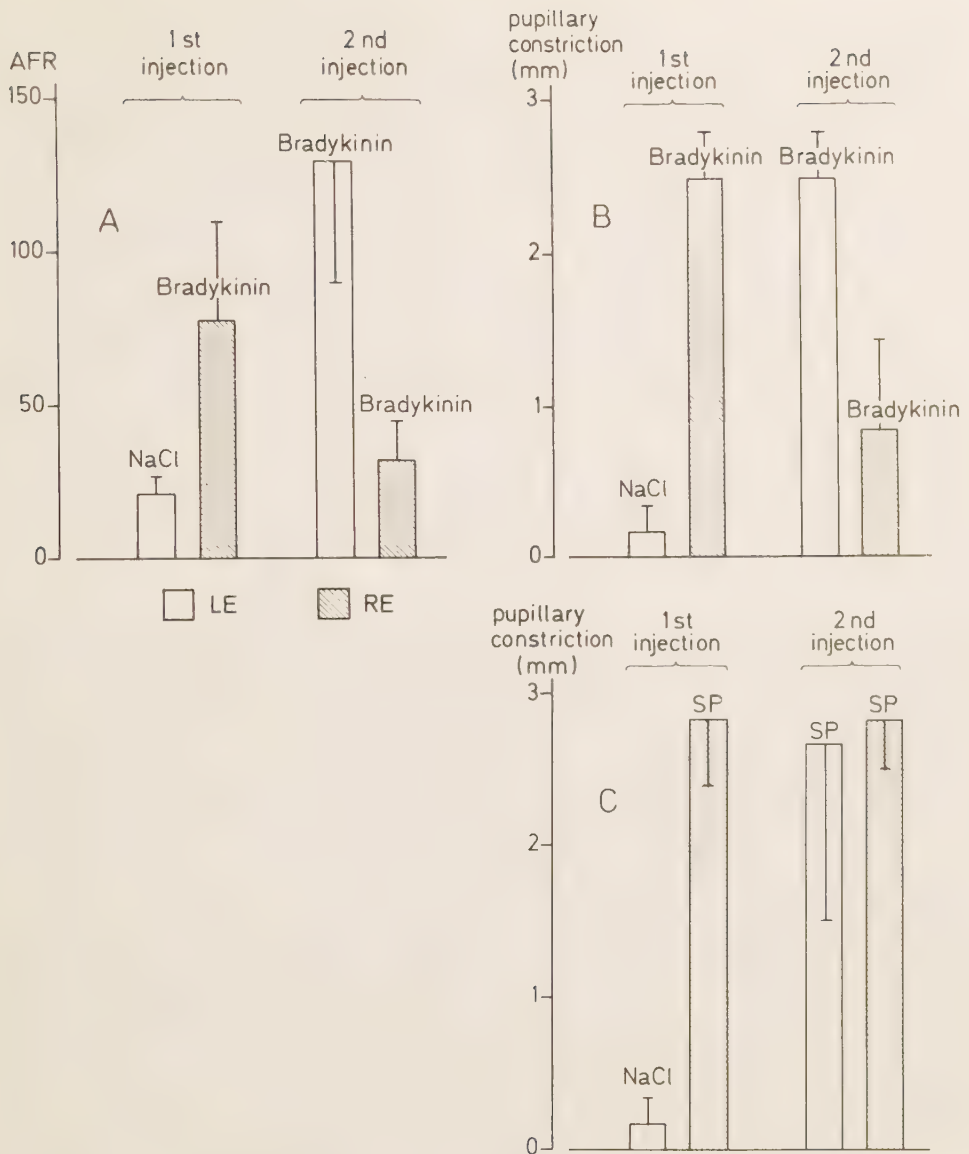


Fig. 2. The AFR and miotic response (pupillary constriction in mm) to intra-vitreous injections of 350 pmole bradykinin (A, B) or 3 nmole SP (C) (right eye, RE) or 0.9 percent saline (left eye, LE) followed 3 (pupillary constriction) or 5 (AFR) hours later by injection of the same dose of bradykinin or SP into both eyes. Upon repeated injections the eyes became refractory to bradykinin. The difference between the left and right eye was highly significant (Student's t-test, $P < 0.001$) with respect to both the first and the second injection. With SP the eyes remained responsive to the second injection. The SP dose tested was too small to have more than marginal effect on AFR (cf Holmdahl et al., 1981) and only the miotic response was measured. Three rabbits were studied in each group; vertical lines give the standard error of the mean.

with either of the two SP antagonists (10^{-5} M) (Fig. 1), suggesting that SP mediates the bradykinin-evoked response.

The sphincter pupillae muscle responded to electrical stimulation with a slow contraction, reaching a maximum 30-40 s after cessation of stimulation (Fig. 1). Atropine reduced the contractile response to low frequency (5-10 Hz) stimulation by about 25%; responses to high frequency (10-20 Hz) stimulation were not much affected (see also Tornqvist et al., 1982). The atropine-resistant contraction could be abolished by either of the two SP antagonists, indicating that the noncholinergic contraction is mediated

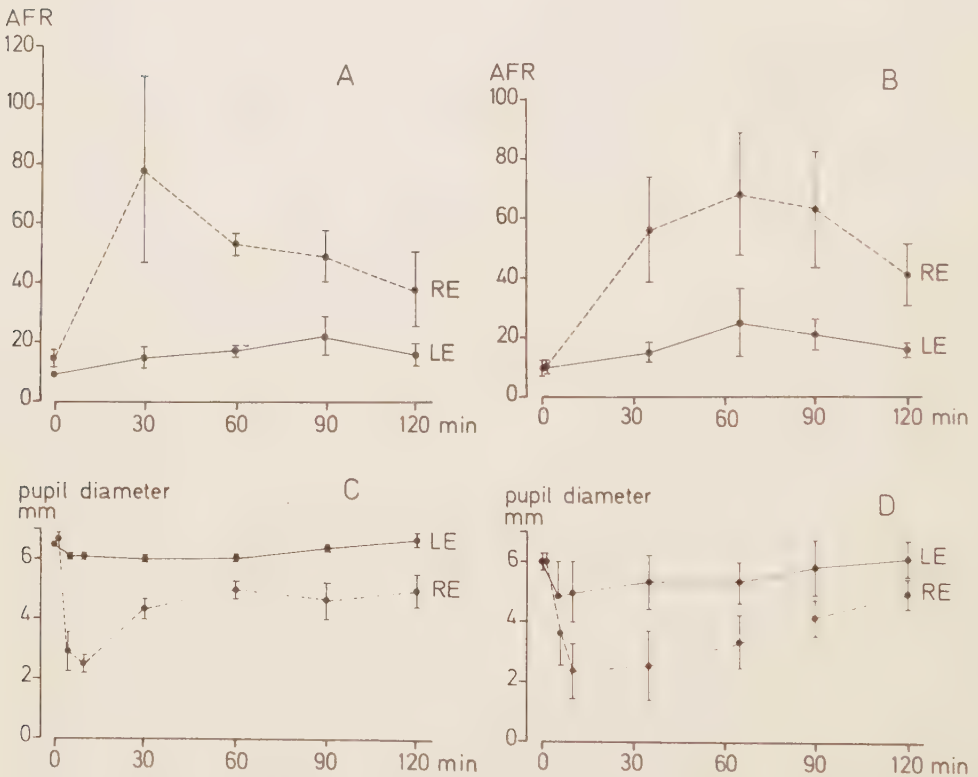


Fig. 3. The AFR (A, B) and the pupil diameter (mm) (C, D) in response to intravitreal injection of 350 pmole of bradykinin into both eyes 4 hours after the intravitreal injection of 15 nmole of TTX (A, C) or 1 hour after topical application of 90 nmole of Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH₂ (B, D) into the left eye (LE) and 0.9 percent saline into the right eye (RE). The difference between the left eye and right eye following injection of bradykinin was highly significant in both series of experiments (analysis of variance, $p < 0.01$ in A, $p < 0.001$ in B-D). Three rabbits were tested in each series; vertical lines give the standard error of the mean.

by SP (Fig. 1). Interestingly, pretreatment with bradykinin, but not with SP, lowered the contractile response to electrical stimulation (Fig. 1). This was a dose-dependent effect, the maximal reduction was approx. 50% and the maximally effective concentration of bradykinin was 10^{-8} M.

Intravitreal injection of bradykinin or SP produced AFR and miosis. Repeated administration of bradykinin 3 or 5 hours later evoked a much reduced response (Fig. 2). In contrast, the response to repeated administration of SP was unchanged. Topical application of atropine 1 h before injection of bradykinin did not prevent either the miotic response or the AFR, whereas intravitreal injection of TTX completely abolished the response to bradykinin (Fig. 3) without affecting the response to SP (not shown). In itself, topical application of the two SP antagonists had no effects on the eye but the inflammatory response to the subsequent injection of bradykinin (or SP) was greatly suppressed (Fig. 3).

Discussion

Like SP, bradykinin is known to cause inflammation in the eye (Cole & Unger, 1974; Eakins, 1977), characterized by constriction of the pupil (miosis), hyperemia, and breakdown of the blood-aqueous barrier, thereby mimicking the response to trauma. Bradykinin and SP act on separate receptors (as illustrated in Fig. 1) and it has been shown previously (Leander et al., 1981) that ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP does not block the bradykinin receptor. Hence, the results of the experiments on the isolated rabbit sphincter pupillae muscle suggest that bradykinin acts by releasing neuronal SP, that the releasable SP pool can be exhausted by bradykinin, and that the bradykinin-sensitive SP pool only partly corresponds to that which can be released by electrical stimulation. Similarly, the results of the studies on the bradykinin-evoked inflammatory response in the rabbit eye indicate that the symptoms are caused by neuronal SP released by bradykinin and that bradykinin is capable of exhausting a neuronal pool of SP.

The inflammatory response in the eye is thought to depend upon agents released from peripheral sensory nerve fibres (Butler & Hammond, 1977; Holmdahl et al., 1981). SP is a likely candidate for the following reasons: 1) Exogenous SP evokes symptoms of inflammation (Bill et al., 1979; Butler & Hammond, 1980; Holmdahl et al., 1981); 2) An SP-releasing agent (capsaicin) evokes inflammation (Camras & Bito, 1980; Bynke, 1982); 3) SP is a constituent of sensory nerve fibers (Otsuka & Konishi, 1977), probably including those of the trigeminal nerve (Törnqvist et al., 1982); 4) section-

ning of the trigeminal nerve results in suppression of inflammation (Butler & Hammond, 1980; Butler et al., 1981); 5) specific antagonists to SP suppress inflammation (Holmdahl et al., 1981).

The present results support the view that neuronal SP plays a key role in the inflammatory process and emphasize the potential therapeutic usefulness of SP antagonists.

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A specific substance P antagonist blocks smooth muscle contractions induced by non-cholinergic, non-adrenergic nerve stimulation

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Nerve fibres containing substance P (SP) are widely distributed in the body¹⁻³ and seem to innervate autonomic ganglia, blood vessels, epithelial structures and smooth muscle. SP stimulates secretion from exocrine glands, causes vasodilation and contracts non-vascular smooth muscle⁴. The presence of SP in primary sensory neurones has lent support to the view that it is associated with sensory nerve conduction, conceivably as a transmitter⁵, and that it is a causative factor in the 'irritative' response to antidromic stimulation of sensory nerves⁶. Gut smooth muscle contracts in response to non-cholinergic, non-adrenergic nervous stimulation^{7,8}, and it has been suggested that SP acts as an excitatory transmitter in intramural neurones in the gut wall^{2,3,9,10}. Recently, a series of synthetic analogues of SP with antagonist activity to SP has been developed^{11,12}. We report here that a new analogue, (D-Pro², D-Trp^{7,9})-SP, exercises a specific, possibly competitive antagonism to SP. While being a partial agonist it antagonized the contractile response to applied SP and to non-cholinergic, non-adrenergic nerve stimulation in the isolated guinea pig taenia coli and rabbit iris sphincter pupillae muscle, suggesting that SP, or a closely related peptide, is indeed a motor excitatory transmitter. In contrast, (D-Pro², D-Trp^{7,9})-SP did not inhibit the contractile response to non-cholinergic, non-adrenergic nerve stimulation of smooth muscle from the guinea pig urinary bladder.

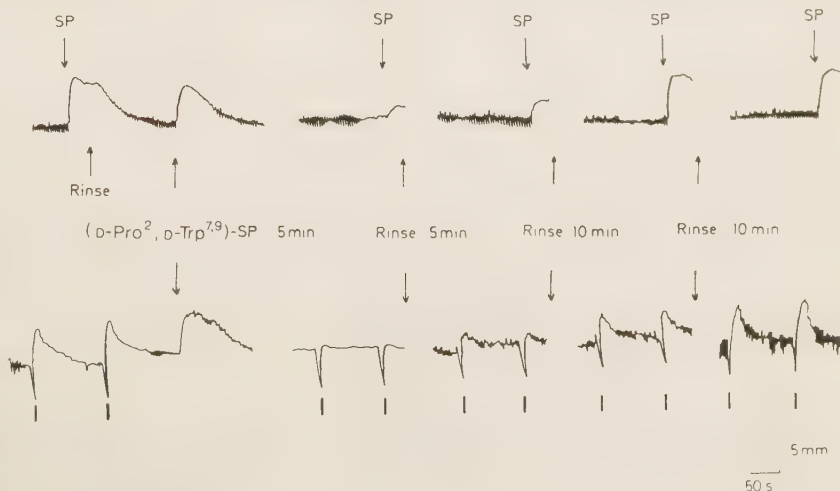
Guinea pig taenia coli preparations, consisting of longitudinal smooth muscle with the attached myenteric plexus¹³, were

placed in Krebs solution, stored at 4°C for ~1 h and then mounted vertically on a Perspex holder in a 7-ml organ bath thermostated at 37°C. One end was attached to a rigid support and the other to a lever connected via a spring to a Grass FT03 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. The load on the muscle was set at 0.2 g. Platinum ring electrodes were placed around the muscle with a constant electrode distance of 5 mm and the electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (15 V over the electrodes, 0.5-1 ms duration). The muscles were stimulated with trains of pulses lasting 3 s and with a frequency of 1-5 Hz, the resting period between stimulations being at least 2 min. The mechanical activity of the preparation was continuously recorded on a Grass model 7 or model 5 polygraph. The bathing fluid was a modified Krebs solution of the following composition (in mM): NaCl 133, NaHCO₃ 16.3, KCl 4.7, MgCl₂ 1.0, NaH₂PO₄ 1.4, CaCl₂ 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7% CO₂ in O₂ giving a pH of 7.2-7.3.

The sphincter pupillae muscle of the rabbit iris was excised, opened and mounted as described for the taenia. Mucosa-free strips of the detrusor of the guinea pig bladder were prepared as described in detail elsewhere¹⁴. The same procedure as above was used to study the motor activity of the sphincter pupillae and the urinary bladder except that they were placed directly in the bath, experiments with sphincter pupillae muscle were run at 35°C, and the stimulation parameters were 10-20 Hz for 10 s for the sphincter pupillae muscle¹⁵ and a single pulse every 20 s for the urinary bladder. (D-Pro², D-Trp^{7,9})-SP was synthesized by techniques analogous to those described elsewhere¹¹.

The addition of SP and of (D-Pro², D-Trp^{7,9})-SP to guinea pig taenia coli produced a contraction which lasted 120-150 s, the tension subsequently returning to baseline (Fig. 1). As illustrated in Fig. 2a the contractile effect of the SP analogue required high concentrations. The contractile response to exogenous SP was strongly inhibited by pretreatment with (D-Pro², D-Trp^{7,9})-SP (Fig. 1), the blockade lasting for at least 30 min without washing. Washing out the SP analogue resulted in a return of the contractile response to exogenous SP and to electrical stimulation (Fig. 1). The analogue also reduced the contractile response to exogenously applied physalamin and eldoisoin but not that to carbachol, histamine, serotonin, bradykinin, Lys⁸-vasopressin or prostaglandin F_{2α} (Fig. 3). In the presence of (D-Pro², D-Trp^{7,9})-SP, the dose response curve

Fig. 1 Effect of (D-Pro², D-Trp^{7,9})-SP (10⁻⁶ M) on the contractile response to exogenous substance P (SP; Peninsula) (5 × 10⁻⁶ M) (upper panel) and to the electrically evoked contraction (lower panel) of isolated guinea pig taenia coli. The muscles were electrically stimulated in the presence of atropine (10⁻⁶ M; ACO) and guanethidine (5 × 10⁻⁶ M; Sigma) for 3 s with 3 Hz as indicated by black rectangles. Note the agonist effect of (D-Pro², D-Trp^{7,9})-SP, the ensuing blockade of the contractile response to exogenous SP and the reduction of the electrically induced contraction on addition of the SP analogue. Electrical stimulation was applied 5 min after the contractile effect of (D-Pro², D-Trp^{7,9})-SP had subsided. The inhibitory effect of (D-Pro², D-Trp^{7,9})-SP on smooth muscle contractions can be washed out as illustrated by the recovery of the contractile response of the taenia coli to exogenous SP and to electrical nerve stimulation. Rinsings and time intervals between registrations are indicated. The registrations shown are typical examples of four to six experiments



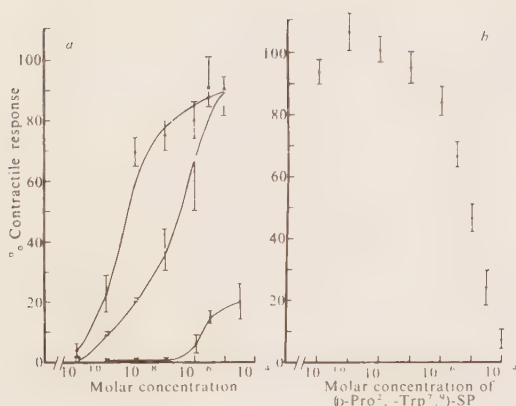


Fig. 2 *a*, Dose-response curves showing the effects of SP alone ($\blacktriangle$), of SP in the presence of (D-Pro², D-Trp^{7,9})-SP ($\circ$), and of (D-Pro², D-Trp^{7,9})-SP alone ($\bullet$) on the tension of the guinea pig taenia coli, expressed as percentage of the contraction induced by carbachol (10^{-5} ; Merck-Darmstadt). In the presence of (D-Pro², D-Trp^{7,9})-SP (10^{-5} M) the dose-response curve for SP was shifted 2.54 log units to the right, suggesting competitive inhibition. The dose-response curves were constructed by adding step wise increasing amounts of peptides to the bath. Below 10^{-7} M three concentrations of SP were tested on each muscle; above 10^{-7} M only one concentration was tested per muscle. The corresponding concentration for the SP analogue was 10^{-6} M. SP was added in a volume of 200 μ l, the SP analogue in 40 μ l. Between each addition the bath was rinsed until the tension had returned to base line (usually three rinses, in all 5–10 min). Each value is the mean of 3–10 experiments. Vertical bars give s.e.m. *b*, Effect of increasing concentrations of (D-Pro², D-Trp^{7,9})-SP on the contractile response of the isolated guinea pig taenia coli to electrical stimulation (3–5 Hz, 3 s). Atropine (10^{-6} M) and guanethidine (5×10^{-6} M) were present in the bath. New muscles were set up for each concentration tested. Electrical stimulation was applied 5 min after the contractile response to the SP analogue had subsided. The results are expressed as per cent of the contractile responses before adding the SP analogue. Each value is the mean of 4–18 experiments. Vertical bars give s.e.m.

for SP was shifted to the right in a manner suggesting competitive inhibition (Fig. 2a). Desensitization with SP is a well known phenomenon⁹, but it can be ruled out as an explanation for the inhibitory effect of the SP analogue because high concentrations of SP ($>10^{-6}$ M) could overcome the inhibition, very high concentrations ($>10^{-5}$ M) giving a maximal contractile response.

Electrical stimulation (1–5 Hz, 3 s) of the taenia elicited a strong contraction, and this response was not affected by addition of (D-Pro², D-Trp^{7,9})-SP. Following addition of the muscarinic blocker atropine (10^{-6} M) and the anti-adrenergic drug guanethidine (5×10^{-6} M), the taenia responded to low-frequency stimulation (1 Hz, 3 s) by relaxing. With higher-frequency stimulation (3–5 Hz, 3 s), the initial relaxation was followed by a contraction on cessation of stimulation¹⁰. Both these responses were inhibited by tetrodotoxin, indicating a neuronal mediation¹⁶. Although relaxation was unaffected, the amplitude of the contraction was greatly reduced by pretreatment with (D-Pro², D-Trp^{7,9})-SP (Fig. 1). Interestingly, the dose-response curve revealed that a 10^{-4} M concentration of (D-Pro², D-Trp^{7,9})-SP reduced the contraction by more than 90% (Fig. 2b).

The iris sphincter pupillae muscle responded to both SP and electrical stimulation with a slow contraction, in the latter case reaching a maximum 30–40 s after cessation of stimulation. The electrically induced contraction lasted for ~150 s. Cholinergic and adrenergic blockade had very little effect on the response to electrical stimulation, but (D-Pro², D-Trp^{7,9})-SP caused a dramatic reduction in the contractile response (Fig. 4a). The remaining contraction was further reduced by adding tetrodo-

toxin. As in the taenia coli, the response of the iris sphincter muscle to exogenous SP, physalaemin and eledoisin (but not that to carbachol) was greatly inhibited by pretreatment with (D-Pro², D-Trp^{7,9})-SP.

The guinea pig urinary bladder responded to both SP and electrical stimulation with a contraction. Cholinergic and adrenergic blockade had little or no effect on the response to electrical stimulation¹⁴. Also, (D-Pro², D-Trp^{7,9})-SP failed to inhibit the response, which could be completely abolished by tetrodotoxin (Fig. 4b).

The results of these experiments indicate that (D-Pro², D-Trp^{7,9})-SP is a partial agonist, exercising a specific, possibly competitive antagonism to SP. Interestingly, (D-Pro², D-Trp^{7,9})-SP also blocked the effects of physalaemin and eledoisin. Together these peptides have been grouped under the name tachykinins^{17,18}; they have the carboxy-terminal three amino acids in common and display smooth muscle stimulating effects^{17,18}. Following cholinergic and adrenergic blockade, electrical nerve stimulation still induced a contraction of the guinea pig taenia coli, urinary bladder and rabbit sphincter pupillae muscle. In the taenia coli and sphincter pupillae muscle these contractions were greatly reduced by (D-Pro², D-Trp^{7,9})-SP, suggesting that SP is involved as a motor excitatory transmitter. However, the SP analogue did not inhibit the contractile response to electrical nerve stimulation of the urinary bladder following cholinergic and adrenergic blockade. Hence, SP is not in all tissues the only non-cholinergic, non-adrenergic motor excitatory neurotransmitter. SP nerve fibres in the smooth muscle of the taenia coli probably originate from cell bodies in the mesenteric plexus^{10,19–21}, but their precise nature and mode of action remain to be established. Our results suggest that they are engaged in an efferent motor excitatory capacity although it cannot be excluded that SP is released as a result of antidromic nerve stimulation. The SP fibres in the sphincter pupillae muscle

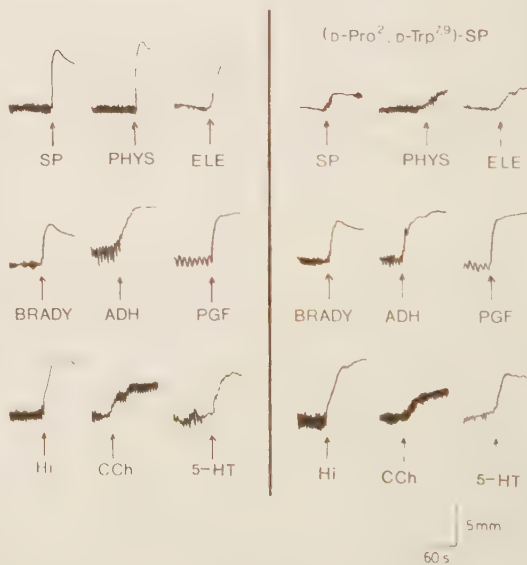
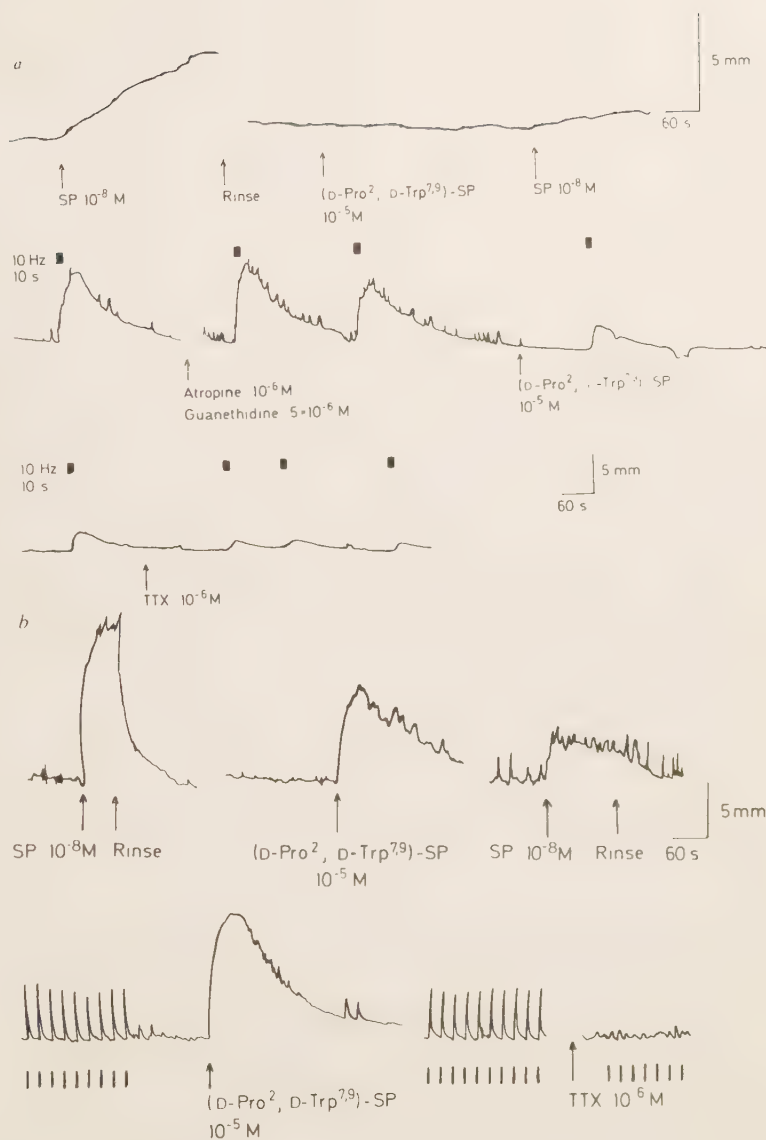


Fig. 3 Contractile response of the isolated guinea pig taenia coli to physalaemin (PHYS), eledoisin (ELE; both Peninsula) at a concentration of 10^{-9} M, SP bradykinin (BRADY; Beckman), histamine (Hi; Merck-Darmstadt), carbachol (CCh) and serotonin (5-HT; Sigma) at a concentration of 10^{-6} M, Lys⁸-vasopressin (antidiuretic hormone, ADH; Ferring) and prostaglandin F_{2α} (PGF; Sigma) at a concentration of 10^{-7} M, with (right) or without (left) 10^{-4} M (D-Pro², D-Trp^{7,9})-SP in the bath. The contractile effect of the SP analogue was allowed to subside before the other compounds were added, usually 5 min later.

Fig. 4 *a*, Effect of (D-Pro², D-Trp^{7,9})-SP on the contractile response to exogenous SP (upper panel) and to the electrically evoked contraction (middle and lower panels) of the rabbit iris sphincter pupillae muscle. In this preparation the SP analogue *per se* had no contractile effect. Electrically induced contraction persisted in the presence of atropine and guanethidine. Note the absence of contractile effect of exogenous SP and the reduction of the electrically evoked contraction following addition of the SP analogue. Addition of tetrodotoxin (TTX; Sankyo) almost completely abolished the residual contractile response. *b*, Effect of (D-Pro², D-Trp^{7,9})-SP on the contractile response to exogenous SP (upper panel) and to the electrically evoked contraction (lower panel) of the guinea pig urinary bladder. The muscles were electrically stimulated in the presence of atropine (10^{-6} M) and guanethidine (5×10^{-6} M) with a single pulse every 20 s as indicated. Note the agonist effect of (D-Pro², D-Trp^{7,9})-SP, the ensuing blockade of the contractile response to exogenous SP and the lack of effect on the electrically induced contraction on addition of the SP analogue. Electrical stimulation or exogenous SP were applied 5–10 min after the contractile effect of (D-Pro², D-Trp^{7,9})-SP had subsided. Addition of TTX completely abolished the contractile response to the electrical field stimulation. The results shown in *a* and *b* are typical examples from a series of four to six experiments.



are probably derived from the trigeminal nerve^{15,22,23}, which is assumed to have a sensory function. Hence, the SP-mediated contraction is either the result of antidromic nerve stimulation or an indication that the trigeminal nerve carries efferent fibres.

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Short title: Substance P antagonist

THE MECHANISM OF ACTION OF A SUBSTANCE P ANTAGONIST
(D-Pro², D-Trp^{7,9})-SP

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1. A newly synthesized substance P (SP) analogue, (D-Pro², D-Trp^{7,9})-SP, specifically antagonizes the contractile effects of SP on the isolated guinea pig taenia coli. In addition, previous studies had indicated that the SP analogue per se is capable of contracting this preparation. The results of the present study on the guinea pig taenia suggest that the smooth muscle contractions produced by the SP analogue are due to histamine release. No contractions were observed following blockade of histamine H₁-receptors by mepyramine or following pretreatment with the histamine liberating agent compound 48/80.
2. Analysis of the inhibition of SP-induced contraction by the analogue suggests that the inhibition is of the competitive type; pA₂ was calculated to be 6.1.
3. We conclude that (D-Pro², D-Trp^{7,9})-SP is a competitive SP antagonist with histamine-releasing properties.

Introduction A newly synthesized analogue of substance P (SP), (D-Pro², D-Trp^{7,9})-SP, has been found to exercise a specific antagonism to SP (and related tachykinins) on various target organs (Holmdahl et al., 1981; Leander et al., 1981). It inhibits neurally mediated non-cholinergic, non-adrenergic contractions of the isolated guinea-pig taenia coli and rabbit iris sphincter (Leander et al., 1981) and also the inflammatory response to trauma in the eye (Holmdahl et al., 1981). The SP analogue was thought to act as a competitive antagonist to SP (Leander et al., 1981). A partial agonism was tentatively suggested because the analogue produced smooth muscle contraction. However, SP is known to release histamine from mast cells (Johnson & Erdös, 1973; Erjavec et al., 1981) and it was conceivable that release of histamine contributed to the smooth muscle contraction produced by the analogue. The present report provides more detailed information on the mode of action of (D-Pro², D-Trp^{7,9})-SP.

Methods Guinea-pig taenia coli preparations, consisting of longitudinal smooth muscle with the attached myenteric plexus (Burnstock, Campbell & Rand, 1966), were placed in Krebs solution, kept at 4°C for about 1 h and then mounted vertically on a Perspex holder in a 7 ml organ bath thermostated at 37°C. One end was attached to a rigid support and the other to a lever connected via a spring to a Grass FT03 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. The load on the muscle was set at 0.2 g. Platinum ring electrodes were placed around the muscle with a constant electrode distance of 5 mm and the electrodes were connected to a Grass S4C stimulator for field stimulation with square wave pulses (15 V over the electrodes, 0.5-1 ms duration). The preparations were stimulated with trains of pulses lasting 3 sec and with a frequency of 3 Hz, the resting period between stimulations being at least 2 min. The mechanical activity of the preparation was continuously recorded on a Grass model 7 or model 5 polygraph. The bathing fluid was a modified Krebs solution of the following composition (in mM): NaCl 133, NaHCO₃ 16.3, KCl 4.7, MgCl₂ 1.0, NaH₂PO₄ 1.4, CaCl₂ 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7% CO₂ in O₂ giving a pH of 7.2-7.3.

(D₁-Pro², D₁-Trp^{7,9})-SP was synthesized as previously described (Folkers *et al.*, 1981; Rosell *et al.*, 1981) and SP was purchased from Peninsula, San Carlos, Ca., USA. In addition the following drugs were used: Atropine (ACO, Stockholm, Sweden), carbachol (Merck, Darmstadt, FRG), compound 48/80 and guanethidine (Sigma, St. Louis, Mo., USA), mepyramine and metiamide (SKF, Wellwyn Garden City, England), methysergide (Sandoz, Basle, Switzerland).

Electrical stimulation was carried out in the presence of atropine (10⁻⁶ M) and guanethidine (5 x 10⁻⁶ M). The contractile response to SP and to (D₁-Pro², D₁-Pro^{7,9})-SP was examined with or without the histamine H₁-receptor antagonist mepyramine (10⁻⁶ M) in the bath and with or without pretreatment (for 15-30 min) of the taenia coli preparation with the histamine-releasing agent compound 48/80 (30 µg per ml in the bath). Also the effects of the 5-hydroxytryptamine antagonist methysergide (10⁻⁵ M) and the histamine H₂-receptor antagonist metiamide (10⁻⁵ M) were examined.

Dose-response curves were constructed by adding step-wise increasing amounts of SP to the bath. Below 10⁻⁷ M three concentrations of SP were tested on each muscle; at and above 10⁻⁷ M only one concentration was tested per muscle. SP was added in a volume of 200 µl, the SP analogue in 40 µl. Between each addition the bath was rinsed until the muscle tension had returned to base-line (usually three rinses, in all 5-10 min). The responses were expressed as percentage of the contractions produced by carbachol (10⁻⁵ M). By using carbachol rather than maximally effective concentrations of SP the

problem of desensitization can be avoided. PA_2 for ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP was calculated according to Schild (Tallarida et al., 1979).

Results The contractile responses produced by ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP and by small amounts of SP ($<10^{-9}$ M) were reduced upon repeated administration (Fig. 1). With high doses of SP (10^{-7} M and above) the response was not reduced

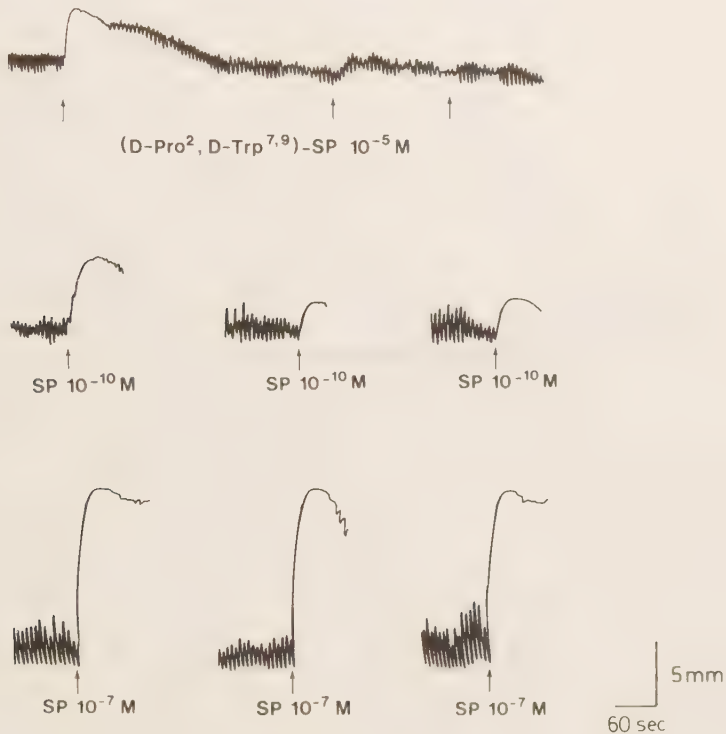


Fig. 1. Contractile responses of the isolated guinea pig taenia coli produced by ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP (top) and by low (10^{-10} M) (middle) and high (10^{-7} M) concentrations of SP (bottom). Repeated administration of ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP and of low concentrations of SP produced a reduced contractile response compared to that observed after the initial administration. The reduction was $56.4\% \pm 8.8$ (mean $\pm$ S.E.M., $n=6$). High concentrations of SP produced the same contractile response upon repeated administrations. Rinsing after each addition of SP was necessary because even with low concentrations of SP the contraction lasted for about 10 min. The registrations shown are typical examples of four to six experiments.

upon repeated administration. The contractile response to ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-
-SP was completely abolished by mepyramine or compound 48/80 (Fig. 2), while

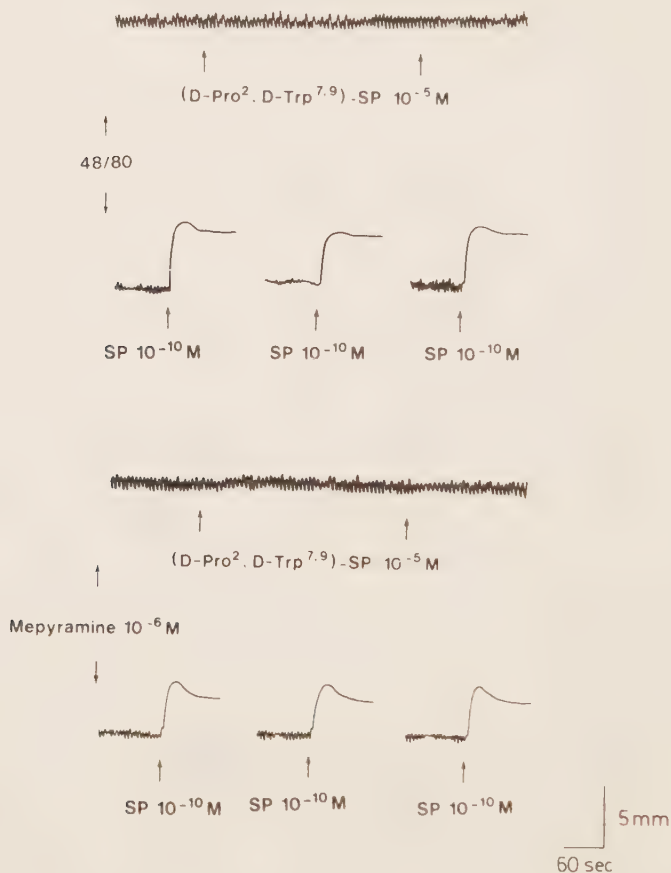


Fig. 2. Pretreatment of the guinea pig taenia coli with the histamine-liberating agent compound 48/80 (top pair of registrations) or with the histamine H_1 -receptor antagonist mepyramine (bottom pair of registrations) abolished the contractions produced by ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP. Under these circumstances low concentrations of SP (10^{-10} M) produced the same contractile response upon repeated administrations. Rinsing after each addition of SP. The mepyramine dose used effectively blocked the effect of histamine (10^{-6} M) (see Fig. 3). The registrations shown are typical examples of four to six recordings.

the initial and subsequent responses to small doses of SP became identical. The response to high doses of SP was unaffected by mepyramine and by compound 48/80 (not shown in fig.). The addition of mepyramine or compound 48/80 to the bath did not affect the tension of the smooth muscle (not shown). Neither methysergide nor metiamide affected the contractile responses produced by the two peptides, and metiamide was without effect on the contractile response to histamine (not shown). Mepyramine blocked the histamine-induced contraction (Fig. 3) but was without effect on the contractile response to 5-hydroxytrypt-

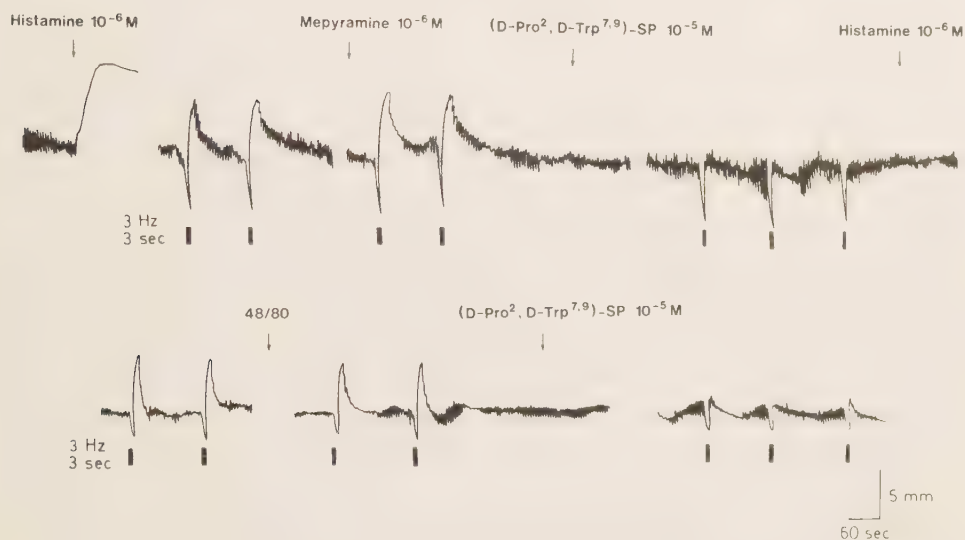


Fig. 3. Effect of histamine and of electrical stimulation (15 V, 3 Hz, 3 s) on the tension of the taenia with atropine (10^{-6} M) and guanethidine (5×10^{-6} M) in the bath. Addition of the histamine H_1 -receptor antagonist mepyramine (top) abolished the response to histamine but not to electrical stimulation. Subsequent addition of (D-Pro², D-Trp^{7,9})-SP abolished the contractile response to electrical stimulation, leaving only the relaxation. Administration of the histamine-liberating agent compound 48/80 (below) did not affect the response to subsequent electrical stimulation, whereas addition of (D-Pro², D-Trp^{7,9})-SP abolished the contraction. The results shown are typical examples from a series of four to six experiments.

amine, whereas methysergide blocked the response to 5-hydroxytryptamine but not that to histamine (not shown).

Electrical stimulation of the taenia coli in the presence of atropine and guanethidine produced relaxation followed by contraction upon cessation of stimulation. The response to electrical stimulation was not affected by addition of mepyramine or pretreatment with compound 48/80. The contraction could be abolished by ($\underline{\underline{D}}$ -Pro², $\underline{\underline{D}}$ -Trp^{7,9})-SP, leaving only the relaxatory response (Fig. 3).

($\underline{\underline{D}}$ -Pro², $\underline{\underline{D}}$ -Trp^{7,9})-SP inhibited the contractile response produced by SP and shifted the dose-response curve of SP to the right, the pA_2 being 6.1 (Fig. 4). The estimated slope of the Schild plot (Tallarida et al., 1979) was -1.0.

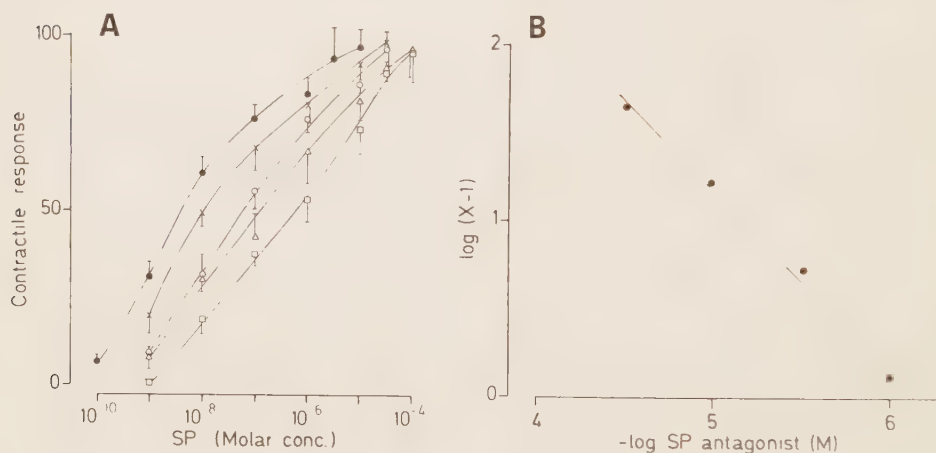


Fig. 4. A. Dose-response curves showing the effects of SP alone (●) and of SP in the presence of increasing concentrations of ($\underline{\underline{D}}$ -Pro², $\underline{\underline{D}}$ -Trp^{7,9})-SP (x, 10^{-6} M; o 3×10^{-6} M; ▲, 10^{-5} M; □, 3×10^{-5} M) on the tension of the guinea pig taenia expressed as percentage of the contraction produced by carbachol (10^{-5} M). Each value is the mean of 4-40 experiments. Vertical bars give S.E.M. The results were identical in the presence of 10^{-6} M mepyramine (not shown) except that the contractile response to 10^{-10} M SP was somewhat lower. B. A Schild plot embodying the results of experiments with four concentrations of ($\underline{\underline{D}}$ -Pro², $\underline{\underline{D}}$ -Trp^{7,9})-SP. x is the dose ratio (Tallarida et al., 1979). pA_2 (the intercept on the abscissa) is 6.1. The slope of the line was calculated to be -1.0, $r = 0.997$. The slope had a 95% confidence range of -0.77 to -1.28, giving a 95% confidence range for A_2 of 3.92×10^{-7} to 1.28×10^{-6} M.

Discussion SP, which is known to release histamine from mast cells (Johnson & Erdös, 1973), probably releases histamine also in the isolated taenia coli preparation, since histamine H_1 -receptor blockade or pretreatment with the mast-cell-degranulating and histamine-releasing agent compound 48/80 reduced the contractile response to low concentrations of SP. The contractile response to high concentrations, however, were notably unaffected by H_1 -receptor blockade and histamine depletion. Blockade of histamine H_2 -receptors and 5-hydroxytryptamine receptors was without effect. Interestingly, histamine release thus seems to play a role in the contractile response only to small doses of SP; with large doses of SP the contribution made by histamine was relatively unimportant. Also the SP analogue, ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP, seems to have the capacity to release histamine since the contractile response to this agent at all dose levels could be completely abolished by mepyramine or compound 48/80 pretreatment. Previously, ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP was described as a partial agonist (Leander et al., 1981). The present results suggest that the contraction produced by this SP analogue is due to histamine release rather than to interaction with SP receptors on the smooth muscle.

The SP antagonism displayed by ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP on taenia coli was analyzed by constructing SP concentration-response curves in the absence or presence of the SP analogue. The results show a parallel shift to the right of the dose-response curve with increasing concentrations of the SP analogue. The slope of the Schild plot conformed to the theoretical value for competitive inhibition (-1), supporting the view that the inhibition is competitive in nature (Leander et al., 1981).

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Substance P antagonists release histamine from peritoneal mast cells

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Recently, a series of substance P (SP) analogues have been found to block the action of SP in a specific and competitive manner (Folkers et al., 1981; Leander et al., 1981; Håkanson et al., 1982). Studies dealing with the effects of these antagonists on electrically induced smooth muscle contractions (Leander et al., 1981), and nociceptive (Åkerman et al., 1982) and inflammatory (Holmdahl et al., 1981; Bynke et al., 1982) reactions have indicated important physiological roles for SP and suggested areas of potential clinical usefulness of SP antagonists. The SP antagonists currently available have low potency and contract smooth muscle in a manner suggesting partial agonism. In view of the fact that SP is capable of releasing histamine from mast cells (Johnson and Erdös, 1973; Erjavec et al., 1981) it occurred to us that the partial agonism observed could reflect effects of histamine released by the SP antagonists rather than direct effects of the SP antagonists on SP receptors of smooth muscle.

In order to test this hypothesis we measured the histamine released from peritoneal mast cells upon exposure to SP and two SP analogues with antagonistic properties, (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP and $\text{Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH}_2$, the pA_2 values being 6.1 (Håkanson et al., 1982) and 6.5 (Leander et al., to be published); respectively. SP was purchased from Peninsula, San Carlos, CA, USA. The SP analogues were synthesized by methods analogous to those previously described (Folkers et al., 1981). Adult male Sprague-Dawley rats (freely fed, body weight 300-350 g) were killed by decapitation under light diethyl ether anaesthesia. Peritoneal cells, consisting of 3-6% mast cells (Padaver, 1963; Kurose and Saeki, 1981), were obtained by peritoneal lavage with 10 ml buffered salt solution (BSS), containing 145 mM NaCl, 2.7 mM KCl and 10% (v/v) Sörensen phosphate buffer ($\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$, 67 mM, pH 6.8). After gentle abdominal massage for 3-4 min, the intra-abdominal mast cell-rich fluid was removed with a pipette. Abdominal fluid from 2 rats was pooled and centrifuged at approximately $300 \times g$ for 10 min at room temperature. The precipitated cells were resuspended in 10 ml BSS to which was added 1 mg/ml of bovine serum albumin (Armour, Kankakee, IL, USA) (BSSA). The cell suspension was washed twice in the BSSA. Finally the cells

were suspended in 30 ml BSSA at room temperature and aliquots of 0.9 ml were transferred to plastic test tubes and preincubated for 5 min at 30°C before adding the peptide in a volume of 0.1 ml BSS. The mixture was incubated for 5 min at 30°C and the reaction was interrupted by placing the samples in ice. They were then centrifuged at 600 x g for 5 min at 0°C. Aliquots (0.1 ml) of the supernatant were taken for direct fluorometric assay of histamine (Håkanson and Rönnerberg, 1974). The sediment was taken up in 1 ml redistilled H₂O, centrifuged for 5 min at 0°C and 0.1 ml of the supernatant was assayed for histamine. All determinations were made in duplicate. The amount of histamine released was expressed as per cent of the total amount of histamine present in the peritoneal cells at the start of the experiment. The results were corrected for 4-5% spontaneous release.

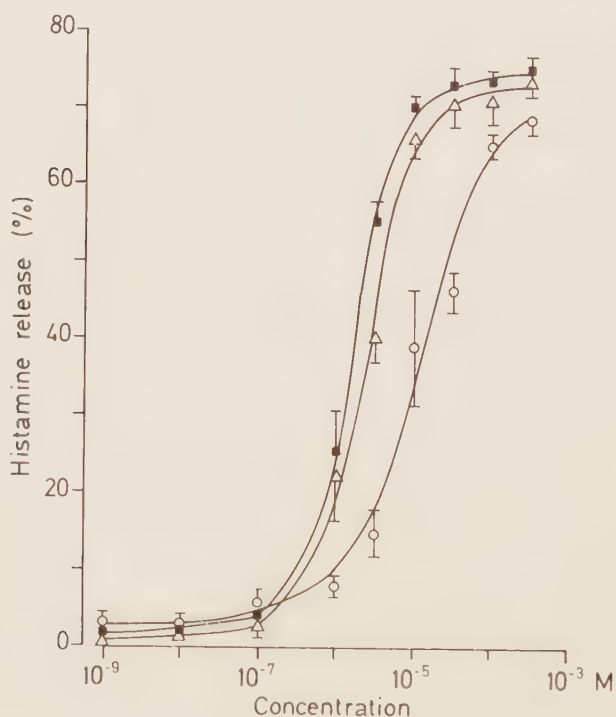


Fig. 1. Release of histamine from rat peritoneal mast cells by substance P (SP, O) and by two SP antagonists (D-Pro^2 , $\text{D-Trp}^{7,9}$)-SP (■) and $\text{Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH}_2$ (Δ). Ordinate: Release in percent of the total histamine minus the histamine release in the absence of peptides. Values are means of 3-4 separate determinations. Vertical lines give S.E.M.

SP and the two SP analogues were fairly potent in releasing histamine (Fig. 1), the analogues even more so than SP in that they produced significant histamine release at lower concentrations than did SP. The ED_{50} value for SP was 1.3×10^{-5} M and the corresponding values for ($\underline{D}$ -Pro², $\underline{D}$ -Trp^{7,9})-SP and Arg-Gln- $\underline{D}$ -Trp-Phe- $\underline{D}$ -Trp-Leu-Met-NH₂ were 1.6×10^{-6} M and 2.5×10^{-6} M, respectively.

The findings support the assumption made in a previous report (Håkanson et al. 1982) that the SP analogues cause smooth muscle contraction at least partly through release of histamine from mast cells in the smooth muscle preparations. This assumption was based on the observation that the two SP analogues did not contract the isolated guinea pig taenia coli in the presence of histamine H₁-receptor antagonists or following pretreatment with the histamine-releasing, mast cell-degranulating compound 48/80 (Håkanson et al. 1982). The design of clinically useful SP antagonists will probably therefore require not only improvements in antagonistic potency but also elimination of histamine-releasing activity.

Acknowledgements

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